PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 5/10, A01H 5/00, A01N 63/02

A2

(11) International Publication Number: WO 97/06268

(43) International Publication Date: 20 February 1997 (20.02.97)

(21) International Application Number: PCT/GB96/01846

(22) International Filing Date: 29 July 1996 (29.07.96)

(30) Priority Data:
9516241.8 8 August 1995 (08.08.95) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB). PAINE, Jacqueline, Ann, Mary [GB/GB]; 5 Frensham, Crown Wood, Bracknell, Berkshire RG12 0TQ (GB).

(74) Agents: ROBERTS, Alison, Christine et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: DNA CONSTRUCTS

(57) Abstract

A chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the *alc*R gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

DNA CONSTRUCTS

The present invention relates to DNA constructs and plants incorporating them. In particular, it relates to promoter sequences and their use in the expression of genes which confer insecticidal activity on plants.

Advances in plant biotechnology have resulted in the generation of transgenic plants which are protected against feeding insect larvae.

Many organisms produce proteins which are harmful to insects and among these is the organism *Bacillus thuringiensis* which produces a crystal-associated protein δ endotoxin which kills insect larvae upon ingestion. It is not, however, toxic to mammals. It is thus very useful as an agricultural insecticide. Many strains of *B. thuringiensis* are active against insect pests, and the genes encoding for the insect endotoxins have been characterised. The *B. thuringiensis* δ endotoxins include those specifically insecticidal to Lepidopteran larvae (such as the CryI type proteins), those specifically insecticidal to Coleopteran larvae (such as the CryIII type proteins) and those with dual specificity for Lepidoptera and Coleoptera (such as CryV). Chimeric proteins comprising at least part of a *B. thuringiensis* endotoxin have also been proposed with the aim of improving the properties of the endotoxin in some way, for example improved speed of kill. Transgenic plants expressing genes which encode for the insecticidal endotoxins are also known.

Other ways of damaging insects include stimulating plant metabolic pathways which produce metabolites which are insecticidal.

We propose a system where genes encoding active insecticidal proteins such as *B. thuringiensis* endotoxins would be expressed in an inducible manner dependent upon application of a specific activating chemical. Alternatively, the induction of pathways which produce metabolites damaging to insects could be acheived. This approach has a number of benefits, including the following:

1. Constitutive expression in plants of insect resistance genes such as *B. thuringiensis* endotoxins, will lead to a significant increase in the selection pressure for resistant insect species. The inducible regulation of insect resistance genes will reduce the risk of development of resistant pests. For example, insecticidal gene expression can be induced only at the point in the growing season where protection is required. In addition,

switchable insect tolerance can be used as a part of an integrated pest management system, in which chemical treatments to induce insecticidal gene expression can be alternated with standard insecticidal pesticide treatments.

2. There is a risk that overexpression, from strong constitutive promoters, could lead to detrimental effects on plant development resulting in aberrant germination flowering or yield penalties. Inducible expression would reduce the risk of detrimental effects as the transgene could be expressed for a short period avoiding sensitive points in development.
3. The switch chemical could be added to standard insecticide formulations to give both a chemical and gene effect, thus killing insects by two independent mechanisms.

We have developed an inducible gene regulation system (gene switch) based on the *alc*R regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alc*A promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

The *alc*A/*alc*R gene activation system from the fungus *Aspergillus nidulans* is also well characterised. The ethanol utilisation pathway in *A. nidulans* is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes *alc*A and *alc*R have been shown to lie close together on linkage group VII and *ald*A maps to linkage group VIII (Pateman JH *et al*, 1984, *Proc. Soc. Lond.*, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, *Curr. Genet.* 8:253-259). Gene *alc*A encodes ADHI in *A. nidulans* and *ald*A encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both *alc*A and *ald*A are induced by ethanol and a number of other inducers (Creaser EH *et al*, 1984, *Biochemical J.*, 255:449-454) *via* the transcription activator *alc*R. The *alc*R gene and a co-inducer are responsible for the expression of *alc*A and *ald*A since a number of mutations and deletions in *alc*R result in the pleiotropic loss of ADHI and aldDH (Felenbok B *et al*, 1988, *Gene*, 73:385-396; Pateman *et al*, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from *alc*A by binding to three specific sites in the *alc*A promoter (Kulmberg P *et al*, 1992, J. Biol. Chem, 267:21146-21153).

The *alc*R gene was cloned (Lockington RA *et al*, 1985, *Gene*, 33:137-149) and sequenced (Felenbok *et al*, 1988). The expression of the *alc*R gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN,

1975, *Eur. J. Biochem.* 51:573-577; Lockington RA *et al*, 1987, *Mol. Microbiology*, 1:275-281; Dowzer CEA and Kelly JM, 1989, *Curr. Genet.* 15:457-459; Dowzer CEA and Kelly JM, 1991, *Mol. Cell. Biol.* 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P *et al*, 1991, *FEBS Letts.*, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure containing two Zn(II) atoms (Pan T and Coleman JE, 1990, *Biochemistry*, 29:3023-3029; Halvorsen YDC *et al*, 1990, J. Biol. Chem, 265:13283-13289). The structure of ALCR is similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P *et al*, 1992, *Mol. Cell. Biol.*, 12:1932-1939).

The regulation of the three genes, *alc*R, *alc*A and *ald*A, involved in the ethanol utilisation pathway is at the level of transcription (Lockington *et al*, 1987; Gwynne D *et al*, 1987, *Gene,* 51:205-216; Pickett *et al*, 1987, *Gene,* 51:217-226).

There are two other alcohol dehydrogenases present in *A. nidulans*. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by *alc*B and is also under the control of *alc*R (Sealy-Lewis & Lockington, 1984). A third alcohol dehydrogenase has also been cloned by complementation with a adh- strain of *S cerevisiae*. This gene *alc*C, maps to linkage group VII but is unlinked to *alc*A and *alc*R.

The gene, *alc*C, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL *et al*, 1985, *EMBO J.*, 4:2094-2099). ADHIII has been shown to be involved in the survival of *A. nidulans* during periods of anaerobic stress. The expression of *alc*C is not repressed by the presence of glucose, suggesting that it may not be under the control of *alc*R (Roland LJ and Stromer JN, 1986, *Mol. Cell. Biol.* 6:3368-3372).

In summary, *A. nidulans* expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene *alc*A only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alc*R gene and constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the *alc*A gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADH1 enzyme are

produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alc*A gene and its product, ADH1, are not expressed in the absence of inducer. Expression of *alc*A and production of the enzyme is also repressed in the presence of glucose.

Thus the *alc*A gene promoter is an inducible promoter, activated by the *alc*R regulator protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The *alc*R and *alc*A genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, *Gene,* 33:137-149; Felenbok B *et al*, 1988, *Gene,* 73:385-396; Gwynne *et al*, 1987, *Gene,* 51:205-216).

Alcohol dehydrogenase (adh) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of adh genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alc*R regulator protein has been found in any plant. Hence the *alc*R/*alc*A type of gene regulator system is not known in plants. Constitutive expression of *alc*R in plant cells does not result in the activation of endogenous adh activity.

According to a first aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the *alc*R gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

When the target gene encodes an insect-damaging protein, it is advantageous for that protein to be orally active. Examples of orally active insecticidal proteins are *B. thuringiensis* δ endotoxins and therefore, the target gene may encode at least part of a *B. thuringiensis* δ endotoxin.

We have found that the *alc*A/*alc*R switch is particularly suited to drive genes which encode for *B. thuringiensis* endotoxins for at least the following reasons.

The *alc*A/*alc*R switch has been developed to drive high levels of gene expression. In addition, the regulatory protein *alc*R is preferably driven from a strong constitutive promoter

such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.

Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about be expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

We have tested the specificity of *alcA/alcR* system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when various alcohols and ketones listed in Table 1 and used in agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3). This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management be considered as a specific inducer of the *alcA/alcR* gene switch in a field situation.

Table 1

1.	Isobutyl methyl ketone	13.	acetonyl acetone
2.	Fenchone	14.	JF5969 (cyclohexanone)
3.	2-heptanone	15.	N-methyl pyrrolidone
4.	Di-isobutyl ketone	16.	polyethylene glycol
5.	5-methyl-2-hexanone	17.	propylene glycol
6.	5-methylpentan-2,4-diol	18.	acetophenone
7.	ethyl methyl ketone	19.	JF4400 (methylcyclohexanone)
8.	2-pentanone	20.	propan-2-ol
9.	glycerol	21.	butan-2-ol
10.	γ-butyrolactone	22.	acetone
11.	diacetone alcohol	23.	ethanol
12.	tetrahydrofurfuryl alcohol	24.	dH_2O

A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the *alc*A/*alc*R switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, absisic acid, auxin, gibberelic acid, various agrochemicals, all failed to induce the *alc*A/*alc*R system.

The present invention is not limited to any particular endotoxin, and is also applicable to chimeric endotoxins.

The first promoter may be constitutive, or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alc*R gene, is obtainable from *Aspergillus nidulans*, and encodes the *alc*R regulator protein.

The inducible promoter is preferably the *alc*A gene promoter obtainable from *Aspergillus nidulans* or a "chimeric" promoter derived from the regulatory sequences of the *alc*A promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The *alc*A promoter or a related "chimeric" promoter is activated by the *alc*R regulator protein when an alcohol or ketone inducer is applied.

The inducible promoter may also be derived from the *ald*A gene promoter, the *alc*B gene promoter or the *alc*C gene promoter obtainable from *Aspergillus nidulans*.

The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alc*A/*alc*R-derived cassette include those listed by Creaser *et al* (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cylcohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

The invention further provides a method for controlling plant gene expression comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to a regulator sequence which is derived from the *alc*R gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes for a *B. thuringiensis* δ endotoxin, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

Various preferred features and embodiments of the present invention will now be described by way of the following non-limiting examples and the drawings in which:

Figure 1 is a plot showing the time course of induction of AR10 segregating population with 7.5% ethanol;

Figure 2 is a plot showing CAT activity in AR 10-30 homozygous line on root drenching with various chemicals;

Figure 3 is a plot showing CAT activity in AR 10-30 homozygous line on root drenching with various chemicals;

Figure 4 shows the production of a 35S regulator construct;

Figure 5 shows the production of a reporter construct;

Figure 6 illustrates switchable insect resistance vectors;

Figure 7 illustrates the sequence of the optimised CryIa(c) gene;

Figure 8 shows the restriction sites in the optimised CryIa(c) gene;

Figure 9 illustrates the sequence of the Cry V gene;

Figure 10 shows the vector 5129 bps containing the CryV gene;

Figure 11 illustrates the sequence of the vector pMJB1; and

Figure 12 is a map of vector pJRIi.

EXAMPLE 1

Production Of The alcR Regulator Construct.

The alcR genomic DNA sequence has been published, enabling isolation of a sample of alcR cDNA.

The alcR cDNA was cloned into the expression vector, pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter . This promoter is a constitutive plant promoter and will continually express the regulator protein. The nos polyadenylation signal is used in the expression vector.

Figure 4 illustrates the production of the 35S regulator construct by ligation of alcR cDNA into pJR1. Partial restriction of the alcR cDNA clone with BamHI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with BamHI and phosphatased to prevent recircularisation. The alcR gene was thus placed under control of the CaMV 35S promoter and the nos 3' polyadenylation signal in this "35S-alcR" construct.

EXAMPLE 2

Production Of The alcA-CAT Reporter Construct Containing The Chimeric Prom ter.

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the nos transcription terminator (the "35S-CAT" construct).

The alcA promoter was subcloned into the vector pCaMVCN to produce an "alcA-CAT" construct. Fusion of part of the alcA promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The alcA promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the alcA promoter and the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with BamHI and HindIII. The pCaMVCN vector was partially digested with BamHI and HindIII, then electrophoresed so that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into E coli and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

EXAMPLE 3

Gene Constructs

We have generated the following constructs summarised in Figure 6:

Vector 1 contains the enhanced 35S CaMV promoter fused to the tobacco mosaic virus omega sequence translational enhancer (TMV) *Bacillus thuringiensis* Cry I A (c) gene and nopoline synthase (nos) terminator.

Vector 2 is identical to vector 1 with the exception that the *B. thuringiensis* Cry I A (c) gene is replaced with the *B. thuringiensis* CryV gene.

Vector 3 contains the alc R regulatory protein gene from *Aspergillus nidulans* driven from the 35S CaMV promoter, alc A promoter region, TMV enhancer Cry I A (c) and nos terminator.

Vector 4 is identical to vector 3 with the exception of the Cry I A(c) gene is replaced with the CryV gene.

The Cry I A (c) gene is an optimised Lepidotera specific synthetic sequence encoding a *Bacillus thuringiensis* endotoxin and is illustrated in Figures 7 and 8. The sequence was obtained from Pamela Green's laboratory, Michigan State University.

The Cry V gene is a novel *Bacillus thuringiensis* endotoxin entomocidal to Coleopteran and Lepidopteran larvae, and is described in our International Patent Publication No WO90/13651. The Cry V gene is a modified synthetic sequence, optimised for plant code usage and has had RNA instability regions removed. It is illustrated in Figures 9 and 10.

EXAMPLE 4

Vector Preparation

Vector 1 - Constitutive Cry 1A (c)

PCR primers were designed to amplify the TMV omega sequence in pMJB1 (see Figure 9) with the addition of a Sal I site adjacent to the XhoI site (see forward oligonucleotide) and destroying the NcoI site and adding a Sal I and Bgl II sites in the reverse oligonucleotide.

Forward oligonucleotide (SEQ ID NO 1)

Sal I

5' CTACTCGAGTCGACTATTTTTACAACAATTACCAAC 3'

XhoI

Reverse Oligonucleotide (SEQ ID NO 2)

5' CTAGGTACC GTCGAC GGATCCGTAAGATCTGGTGTAATTGTAAATAGTAATTG 3'

KpnI *SalI* *BamHI* *BglII*

A PCR was performed with the forward and reverse primers using pMJBI plasmid DNA on a template. The resultant PCR product was cloned into the pTAg vector (LigATor kit, R&D systems); this was then released with Asp 718 and Xho I digestion and cloned into Xho I/Asp 718 digested pMJBI (Figure 10), to form pMJB3. pMJB1 is based on pIBT 211 containing the CaMV35 promoter with duplicated enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopaline synthase poly (A) signal (nos).

The Cry IA(c) synthetic gene was excised as a BgI II Bam H I fragment and cloned into pMJB3. A fragment containing the enhanced 35 CaMV promoter TMV omega sequence, CryI A (c) and the nos terminator was isolated using Hind III and EcoR I. The resultant fragment was ligated into EcoRI/Hind III cut pJRIi (Figure 12) to generate a Bin 19 based plant transformation vector.

Vector 2 - Constitutive Cry V

pMJB3 was cut with Hind III and a Hind III - EcoRI - Hind III linker was inserted. The resultant vector was then cut with Bam HI and a fragment containing the CryV gene as a Bam HI fragment was inserted. The Cry V gene was orientated using a combination of restriction digestion and sequencing. An EcoRI fragment from the resultant vector, containing the enhanced 35 CaMV promoter, TMV omega sequence, CryV gene and nos terminator, was transferred to JRIRiMCS, a Bin 19 based vector containing the pUC18 multiple cloning site.

Vector 3 - Inducible Cry 1A (c)

pMJB3 containing the Cry 1A(c) gene was cut with Sal I, liberating a fragment containing the TMV omega sequence fused to the Cry 1A(c) gene. The resultant fragment was cloned into Sal I cut palc A CAT and orientated by restriction digest. A fragment containing the alcA promoter fused to the TMV omega sequence, Cry 1A(c) gene and nos terminator was excised using HindIII, and transferred to HindIII digested p35SalcRalcAcat, a Bin 19 based vector containing the 35 CaMV promoter fused to alcR cDNA, with the alcAcat reporter cassette removed on HindIII digestion.

Vector 4 - Inducible Cry V

pMJB3 containing the Cry V gene was cut with Sal I, liberating a fragment containing the TMV omega sequence fused to the Cry V gene. The resultant fragment was cloned into Sal I palcACAT, and orientated by restriction digest and sequence analysis. Two fragments containing the alc A promoter Cry V gene and nos terminator were released by digestion with Hind III. A three way ligation of the two Hind III fragments was performed to insert the alc A Cry V nos cassette into p35alcRalcAcat digested with HindIII to remove the alccat cassette. Correct assembly of the cassette was confirmed by restriction digest, southern blotting and sequence analysis.

EXAMPLE 5

Plant transformation

Leaf transformation by *Agrobacterium*.

The transformation was performed according to the method described by Bevan 1984. 3-4 weeks old sterile culture of tobacco (*Nicotiana tabacum* cv Samsum), grown on MS, were used for the transformation. The edges of the leaves were cut off and the leaves cut into pieces. Then they were put into the transformed *Agrobacterium* cells, containing the pJR1RI plasmid with the insert, suspension (strain LBA 4404) for 20 minutes. The pieces were put on plates containing NBM medium (MS medium supplemented with 1mg/l 6-benzylamino purine (6-BAP), 0.1mg/l naphtalene acetic acid (NAA). After 2 days, explants were transferred to culture pots containing the NBM medium supplemented with carbenicillin (500 mg/l) and kanamycin (100 mg/l). Five weeks later, 1 shoot per leaf disc was transferred on NBM medium supplemented with carbenicillin (200 mg/l) and kanamycin (100 mg/l). After 2-3 weeks, shoots with roots were transferred to fresh medium. If required, 2 cuttings from each shoot were transferred to separate pots. One will be kept as a tissue culture stock, the other one will be transferred to soil for growth in the glasshouse after rooting.

Using this transformation method, the four vectors were introduced into tobacco and kanamycin-resistant primary transformants generated. There were 53 primary transformants generated for constitutive Cry1A(c), 54 for constitutive CryV, 73 for inducible Cry1A(c) and 62 for inducible CryV.

EXAMPLE 6

Leaf DNA extraction for PCR reactions.

Leaf samples were taken from 3-4 weeks old plants grown in sterile conditions. Leaf discs of about 5 mm in diameter were ground for 30 seconds in 200 ul of extraction buffer (0.5% sodium dodecyl sulfate (SDS), 250 mM NaCl, 100 mM Tris HCl (tris(hydroxymethyl) aminomethane hydrochloride), pH 8). The samples were centrifuged for 5 minutes at 13,000 rpm and afterwards 150 ul of isopropanol was added to the same volume of the top layer.

The samples were left on ice for 10 minutes, centrifuged for 10 minutes at 13,000 rpm and left to dry. Then they were resuspended in 100 ul of deionised water. 2.5 ul was used for

the PCR reaction at the conditions described by Jepson *et al* , Plant Molecular Biology Report 9(2), 131-138 (1991).

The primary transgenics generated were tested by PCR analysis to identify plants which contained the full length transgene:

Constitutive Cry1A(c)

Two PCR reactions were carried out for these extracts using the following primer pairs:

TMV1 5'CTA CTC GAG TCG ACT ATT TTT ACA ACA ATT ACC AAC (SEQ ID NO 3)

CRY1A2R 5'CGA TGT TGA AGG GCC TGC GGT A (SEQ ID NO 4)

The PCR conditions were 35 cycles of 95 °C 1.2mins, 62 °C 1.8 mins, 72 °C 2.5 mins and extension of 6 mins at 72 °C.

CRY1A1 5'GCA CCT CAT GGA CAT CCT GAA CA (SEQ ID NO 5)

NOS 5'CAT CGC AAG ACC GGC AAC AG (SEQ ID NO 6)

The PCR conditions were 35 cycles of 95 °C 0.8 mins, 61 °C 1.8 mins, 72 °C 2.5 mins and extension of 6 mins at 72 °C.

Nine primary transformants gave PCR products for both primer sets; these and two PCR negative lines were planted into soil in 7.5" pots in the glasshouse.

Constitutive Cry V

Two PCR reactions were carried out for these extracts using the following primer pairs:

TMV1 (see above)

CryV1R 5'GCT GTA GAT GGT CAC CTG CTC CA (SEQ ID NO 7)

The PCR conditions were 35 cycles of 94 °C 0.8 mins, 64 °C 1.8 mins, 72 °C 2.5 mins and extension of 6 mins at 72 °C.

CRYV1 5'TGT ACA CCG ACG CCA TTG GCA (SEQ ID NO 8)

NOS (see above)

The PCR conditions were 35 cycles of 94 °C 0.8 mins, 58 °C 1.8 mins, 72 °C 2.0 mins and extension of 6 mins at 72 °C.

24 primary transformants gave PCR products for both primer sets; these and seven PCR negative lines were planted into soil in 7.5" pots in the glasshouse.

Inducible Cry1A(c)

Three PCR reactions were carried out for these extracts using the following primer pairs:

ALCR1 5'GCG GTA AGG CTT TCA ACA GGC T (SEQ ID NO 9)

NOS as above

The PCR conditions were 35 cycles of 94 °C 1.0 mins, 60 °C 1.0 mins, 72 °C 1.5 mins and extension of 6 mins at 72 °C.

The primer pairs TMV1/CRY1A2R, CRY1A1/ NOS were used as above.

Forty-five plants gave PCR products for all primer sets; these and two PCR negative lines were planted into soil in 6" pots in the glasshouse

Inducible CryV

Sixty-two primary transformants have been generated but no PCR analysis carried out at present.

EXAMPLE 7

Western blot analysis.

120 mg of leaf from 3-4 weeks old plants grown in sterile conditions were ground at 4°C in 0.06 g of polyvinylpoly-pyrolidone (PVPP) to adsorb phenolic compounds and in 0.5 ml of extraction buffer (1 M Tris HCl, 0.5 M EDTA (ethylenediamine-tetraacetate), 5 mM DTT (dithiothreiol), pH 7.8). Then 200 ml more of extraction buffer were added. The samples were mixed and then centrifuged for 15 minutes at 4°C. The supernatant was removed, the concentration of protein estimated by Bradford assay using the bovine serum albumin (BSA) as standard. The samples were kept at -70°C until required.

Samples of 25 mg of protein with 33% v/v Laemmli dye (97.5% Laemmli buffer (62.5 mM Tris HCl, 10% w/v sucrose, 2% w/v SDS, pH 6.8), 1.5% pyronin y and 1% b-mercaptoethanol) were loaded on a SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel (17.7% 30:0.174 acrylamide:bisacrylamide), after 2 minutes boiling.

Translation products were separated electro-phoretically in the following buffer (14.4% w/v glycine, 1% w/v SDS, 3% w/v Tris Base). Then they were transferred onto nitro-cellulose (Hybond-CÔ, Amersham) using an electroblotting procedure (Biorad unit) in the following blotting buffer (14.4% w/v glycine, 3% w/v Tris Base, 0.2% w/v SDS, 20% v/v methanol) at 40 mV overnight.

Equal loadings of proteins were checked by staining the freshly blotted nitrocellulose in 0.05% CPTS (copper phtalocyanine tetrasulfonic acid, tetrasodium salt) and 12 mM HCl. Then the blots were destained by 2-3 rinses in 12 mM HCl solution and the excess of dye removed by 0.5 M $NaHCO_3$ solution for 5-10 minutes followed by rinses in deionised water. Filters were blocked for 1 hour with TBS-Tween (2.42% w/v Tris HCl, 8% w/v Nacl, 5% Tween 20 (polyxyethylene sorbitan monolaureate), pH 7.6) containing 5% w/v BSA. Then they were washed for 20 minutes in TBS-Tween supplemented with 2% w/v BSA. Indirect immunodetections were performed with a 1:2000 dilution of a Cry I A (c) or Cry V antiserum as first antibody and with a 1:1000 dilution of a rabbit anti-rabbit antiserum as second antibody, associated with the horseradish peroxidase (HRP). Any excess of antiserum was washed with TBS-Tween supplemented with 2% w/v BSA. ECL (enhanced chemiluminescence) detection was performed using the protocols described by Amersham. Any background was eliminated by additional washes of the membranes in the solution mentioned above. The latter one were then subjected to ECL detection.

An estimation of the level of expression of the *B. thuringiensis* gene was performed on the LKB 2222-020 Ultroscan XL laser densitometer (Pharmacia). A helium-neon laser beam (wavelength 633 nm) was scanning on the autoradiograph a band of 2.4 mm width in the middle of the band corresponding to the translation products.

Each peak was characterized by its area, determined by the inner software from the curve of absorbance function of the beam position.

EXAMPLE 8

Northern blot analysis

Total RNA was fractionated on a 1.2% agarose gel containing 2.2.M formaldehyde. After electrophoresis, the RNA was transferred onto Hybond-N membrane (Amersham) by capillary blotting in 20X SSPE. RNA was fixed to membranes using a combination UV strata linking (Stratagene) and baking for 20 minutes at 80°C. cDNA probes excised from pBluescript SK^- by digestion with *Eco*RI, were labelled with a ^{32}PdCTP using a random priming protocol, described by Feinberg and Vogelstein. Prehybridisations were performed in 5X SSPE, 0.1% SDS, 0.1% Marvel (dried milk powder), 100 mg/ml denatured salmon sperm DNA for 4h at 65°C. Hybridizations were achieved in the same buffer containing

labelled probe at 65°C for 12-24h. Filters were washed at 65°C in 3 x SSC 0.1%SDS for 30 mins, and once at 0.5 x SSC 0.1% SDS for 30 mins prior to autoradiography at -80°C.

Insect Feeding trials

The effectiveness of the present invention can be conveniently tested by feeding leaves of transgenic plants containing the constructs of the present invention to insect larvae, both in the presence and absence, as control, of the inducer.

EXAMPLE 9

Primary Screen

A primary screen was performed by removing leaves from the plants and cutting a number of 1 cm^2 leaf pieces. Replicas were placed separately on 0.75% agar and each infested with approximately 10 sterilized *Heliothis virescens* eggs. The leaf discs were covered and incubated at 25°C, 70% RH for 5 days before scoring the effects of larval feeding. Leaf damage was assigned a score ranging from 0 to 2 in 0.5 increments; 2 denoting no leaf damage (full insect feeding protection) and 0 implying the leaf disc was fully eaten.

Leaves from all the constitutive Cry1A(c) tissue culture primary transformants and wild type tobacco were removed and tested for effect on *Heliothis virescens* as described above. The results are shown in Table 2 below:

TABLE 2

Replicas:	PCR+/-	A	B	C	D	E
35SCry1A(c) 1		1	1	2	2	2
2		1	1	1.5	1	1.5
3		0	0	0	0	0
4		1.5	1	1.5	0	1.5
5	PCR +	0	1.5	0	1.5	0
6		1.5	0	1	1.5	1.5
7	PCR +	1.5	1.5	1.5	1.5	1.5
8		2	1.5	1	2	2
9	PCR +	2	2	1.5	2	2
10		1.5	2	1	1	1.5
11		0	1.5	0	0	0
12		0	0	0	0	1
13		2	2	0	0	1
14		1.5	1	0	0	0
15		2	1	2	0	0
16	PCR +	2	2	2	2	1.5

Replicas:	PCR+/-	A	B	C	D	E
17		2	0	0	1	2
18		0	0	0	1.5	2
19	PCR +	1.5	1.5	1.5	0	1.5
20	PCR +	1.5	1.5	2	1.5	1.5
21		1	0	0	0	0
22		0.5	0	0	2	2
23		0	1	0	0.5	0.5
24		1.5	1.5	0	0	0
25		2	2	1	1	2
26		1	0	0	1	1
27		0	1.5	0	1.5	0
28	PCR +	1.5	1.5	1.5	1.5	1.5
29		0	0	1	1	1.5
30		1	0	0	1.5	0
31	PCR +	1	0	1.5	0	1.5
32		2	1	1.5	0	1.5
33		2	2	2	2	2
34		1	0	0	1.5	2
35		0	2	0	1	1.5
36		2	0	2	2	0
37		2	1	1.5	1	1.5
38	PCR +	1.5	1.5	1.5	1.5	2
39		1.5	0	0	0	1
40		1	1	0	0	0
41		0	0	1	1	0
42		2	1.5	1.5	1.5	2
43		1.5	1.5	1.5	1.5	2
44		2	1.5	1.5	1.5	2
45		2	2	1	0	0
46		0	2	0	2	1
47		2	2	2	0	0
wt tobacco		1	2	2	2	0

In typical bioassay experiments wild type (wt) tobacco mainly gave an average score of less than 0.5.

5 EXAMPLE 10

Primary Screen - Retest

Eleven of the glasshouse grown constitutive Cry1A(c) plants and wild type tobacco were retested. This was to demonstrate that constitutive Cry1A(c) plants that had been

growing in soil in glasshouse conditions for three weeks after tissue culture were also showing reduced leaf damage from *Heliothis virescens*.

TABLE 3

Identity	a	b	c	d	e
35SCry1A(c) 6	0.5	0.5	2	2	2
7	2	1	2	2	2
9	0.5	0.5	2	1	0.5
16	2	2	2	2	2
19	2	2	1.5	2	1.5
20	1.5	1	0	0.5	2
28	0.5	1.5	2	2	2
31	0	0	0	0	1.5
33	2	2	2	2	2
38	1	0	1	2	1
42	1	1	1	1	1
wt tobacco	0	0	0	0	1.5

EXAMPLE 11

Primary Screen with CryV Primary Transformants

Leaves from the constitutive CryV primary transformants and wild type tobacco were tested by the method described above. The damage sustained by excised leaf pieces is recorded below in Table 4.

TABLE 4

Identity	PCR+/-	a	b	c	d
35SCryV 1	+	0	0	0	0
2		0	0	0	0
3		1.5	0	1	0
4	+	0	0	0	0
5		0	0	0	0
6		0	0	0	0
7	+	0	0	0	0
8	+	0	0	0	0
9	+	0	0	0	0
10	+	0	0	0	0
11	+	0	0	0	0
12		0.5	1	1	0.5
13	+	0	0	0	0
14	+	0	0	0	0
15	+	0	0	0	0

Identity	PCR+/-	a	b	c	d
16		0	0	0	0
17		0	0	0	0
18		0	0	0	0
19		0	0	0	0
20		0	0	0	0
21		0	0	0	0
22	+	0	0	0	0
23	+	0	0	0	0
24	+	0	0	0	0
25	+	0	0	0	0
26	+	0	0	0	0
27		0	0	0	0
28		1	0	1.5	1
29	+	0	0	0	0
30		0	0	0	0
31	+	0.5	0.5	0.5	0
32	+	0	0	0	0
33		0	0	0	0
34		0	1.5	0	0
35		0	0	0	0
36		0	0	0	0
37		0	0	0	1.5
38		0	0	0	0
39		0	0	0	0
40		0	0	0	0
41	+	0	0.5	0	1.5
42		0	0	0	0
43		0	0	0	0
44		0	0	0	0
45	+	0	0	0	0
46	+	0	0	0	0
47		0	0	0	0
88	+	0	0	0	0
49		0.5	0.5	0	0
50		0	0	0	0
51		1	0	0	1
52		1	0.5	0.5	0.5
wt tobacco		0	0	0	1.5

EXAMPLE 12

Secondary Screen

To verify the data obtained from the primary screen, a secondary assay was performed on transgenic lines on larger leaf pieces using third instar larvae.

Tobacco leaves were cut from the plant and stored on ice for up to one hour. 40mm diameter leaf discs were cut and placed, cuticle side down, on 3% agar in 50mm plastic pots. Third instar *Heliothis zea* reared on LSU artificial diet for five days at 25°C were weighed and infested onto each leaf disc, one per disc. After infestation lids were placed on the pots and they were stored at 25°C under diffuse light. Treatments were assessed after 3 days for mortality, developmental stage and % leaf disc eaten. Larvae were weighed at infestation and after 3 days.

TABLE 5

% LEAF EATEN										
REPLICAS:	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
wt tobacco	20	15	70	20	30	80	30	80	0	95
35SCry1A(c) 7	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
16	<5	<5	<5	<5	10	10	<5	<5	<5	<5
19	<5	10	10	15	10	5	10	<5	<5	20
20	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
28	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
29	20	<5	<5	30	25	25	30	25	25	25
DEVELOPMENTAL STAGE										
wt tobacco	4	3	5	3	3	5	4	5	3	5
35SCry1A(c) 7	3	3	3	3	3	3	3	3	3	3
16	3	3	3	3	3	3	3	3	3	3
19	3	3	3	3	3	3	3	3	3	4
20	3	3	3	3	3	3	3	3	3	3
28	3	3	3	3	3	3	3	3	3	3
29	4	3	3	5	4	4	4	4	3	3
MORTALITY										
wt tobacco	L	L	L	L	L	L	L	L	D	L
35SCry1A(c) 7	L	D	D	L	D	D	D	D	D	D
16	D	D	D	D	L	L	L	L	D	L
19	D	L	L	L	L	L	L	D	L	L
20	D	D	D	D	D	D	L	L	D	L
28	D	L	D	D	D	D	D	L	L	D
29	L	L	D	L	L	L	L	L	L	L

EXAMPLE 13

Inducible insecticidal activity

Forty -five inducible Cry1A(c) PCR positive lines, two PCR negative lines and wt tobacco in 6" pots were root drenched with 100mls of 5% ethanol. 28 hours later 4 replica small leaf pieces were removed and infested with *Heliothis virescens* eggs. The results are shown below (Table 6). Of the 45 lines grown in the presence of ethanol, 66% showed full resistance on the primary screen test to *Heliothis virescens*. To demonstrate that the plants were inducible and not constitutive expressors leaves were removed 8 days later from 7 of the high scoring lines and infested with *Heliothis virescens* eggs. Previous data from a reporter gene driven by the 35SalcRalcA switch promoter showed that CAT protein levels peaked at 24/48 hours and was on the decline after 48 hours (Figure 1). Other data (not shown) demonstrated that no CAT protein was detected 9 days after induction.

Table 7 demonstrates that in the absence of ethanol irrigation mortality levels were found to be comparable to that seen with a wild type control.

TABLE 6

Heliothis virescens on ALC Cry1A(c)					
glasshouse primary transgenics.					
5% ethanol root drench, ~28hours before assay set up.					
identity	PCR+/-	a	b	c	d
ALCCry1A(c) 1	+	1	2	2	2
2	+	2	2	2	2
3	+	0	0	0	1.5
4	+	2	2	2	2
5	+	0.5	0.5	0.5	1
6	-	2	1	0.5	0
7	+	0	2	1.5	2
8	+	2	2	2	2
9	+	2	2	2	2
10	+	2	2	2	2
11	+	2	2	2	2
12	+	2	2	2	2
13	+	2	2	2	2
14	+	2	2	2	2
15	+	2	2	2	2
16	+	2	2	2	2
17	+	2	2	2	2

18	+	2	2	2	2
19	+	2	2	2	2
20	+	2	2	2	2
21	+	0	0	0	1
22	+	0.5	2	0.5	2
23	+	2	2	2	2
24	+	2	2	2	2
25	+	2	2	2	2
26	+	2	2	2	2
27	+	1	2	2	2
28	+	0.5	2	2	2
29	+	2	2	2	2
30	+	2	2	2	2
31	+	2	2	1	2
32	+	2	2	2	2
33	+	1	2	2	2
34	+	2	2	2	2
35	+	1	2	2	2
36	+	2	2	2	1
37	+	0	0.5	0	1
38	-	0.5	0.5	0.5	0.5
39	+	2	2	2	2
40	+	2	2	2	2
41	+	2	2	2	2
42	+	2	2	2	2
43	+	2	2	2	2
44	+	2	2	2	2
45	+	2	2	2	0.5
46	+	2	2	2	2
47	+	1	2	2	2
wt tobacco		0	0.5	0	0.5

TABLE 7

INDUCED							NO INDUCTION					
identity	PCR+/-	a	b	c	d		identity	PCR+/-	a	b	c	d
17	+	2	2	2	2		17	+	0.5	0.5	0.5	1
32	+	2	2	2	2		32	+	1.5	1	1	0.5
39	+	2	2	2	2		39	+	2	2	0.5	0.5
40	+	2	2	2	2		40	+	0.5	0.5	0.5	0.5
41	+	2	2	2	2		41	+	0.5	2	0	1
43	+	2	2	2	2		43	+	0.5	0.5	0.5	0.5
44	+	2	2	2	2		44	+	0.5	0	0.5	0.5
wt tobacco		0	0.5	0	0.5		wt tobacco		0	0	0.5	0.5
	-						wt tobacco		0	1	0	0

Several lines were chosen for a secondary screen to test the effect of induction on insect feeding, along with the constitutive Cry1A(c) line10 and wt tobacco as controls. 10 leaf pieces for each line were removed from primary transformants 12 days after they had been induced by root drenching with 100mls of 5% ethanol and placed on 3% agar in 50 mm pots with lids and incubated overnight at 25°C and 60% humidity. Expression of Cry1A(c) protein was expected to be low or undetectable after 12 days.. The plants were then root drenched with 100mls of 5% ethanol. 22 hours later leaves were excised and ten 40mm leaf pieces were removed and placed on 3% agar in 50mm pots with lids. Five uninduced and 5 ethanol induced leaf discs were infested with 3rd instar *Heliothis zea* and 5 of each infested with *Heliothis virescens* reared as described above. Table 8 demonstrates that wild type controls in the presence or absence of ethanol show a high percentage leaf disc eaten, while the 35S controls show good insect control under both chemical regimes. Transgenic lines containing the Alc Cry IA(c) construct showed poor insect control in the absence of ethanol treatment. Table 8 shows induction with ethanol gives insect control comparable to that seen in the 35S Cry I A (c) control.

TABLE 8

nos 1-5=*H. zea*					
nos 6-10=*H. virescens*					
line		**%eaten**			**%eaten**
wt uninduced	1	45		wt induced 1	55
	2	0		2	55
	3	25		3	95
	4	30		4	50
	5	45		5	25
	6	95		6	25
	7	5		7	55
	8	50		8	30
	9	20		9	20
	10	15		10	25
35S/10 uninduced	1	10		35S/10 induced 1	<5
	2	<5		2	<5
	3	15		3	5
	4	10		4	5

	5	<5		5	5
	6	0		6	<5
	7	<5		7	10
	8	<5		8	<5
	9	<5		9	0
	10	<5		10	5
66 uninduced	1	15		66 induced 1	<5
	2	10		2	<5
	3	0		3	<5
	4	50		4	<5
	5	50		5	10
	6	10		6	0
	7	10		7	<5
	8	15		8	<5
	9	0		9	<5
	10	10		10	<5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 Stanhope Gate
(C) CITY: London
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: DNA CONSTRUCTS

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9516241.8
(B) FILING DATE: 08-AUG-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CATCTCGAGT CGACTATTTT TACAACAATT ACCAAC 36

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTAGGTACCG TCGACGGATC CGTAAGATCT GGTGTAATTG TAAATAGTAA TTG 53

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTACTCGAGT CGACTATTTT TACAACAATT ACCAAC 36

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGATGTTGAA GGGCCTGCGG TA 22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCACCTCATG GACATCCTGA ACA 23

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CATCGCAAGA CCGGCAACAG 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCTGTAGATG GTCACCTGCT CCA 23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGTACACCGA CGCCATTGGC A 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCGGTAAGGC TTTCAACAGG CT 22

CLAIMS

1. A chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which is derived from the *alc*R gene and encodes a regulator protein, and an inducible promoter operatively linked to a target gene which encodes a protein which is damaging to insects or whose expression induces a metabolic pathway which produces a metabolite which is damaging to insects, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

2. A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the target gene encodes an orally active insecticidal protein.

3. A chemically inducible plant gene expression cassette as claimed in claim 2, wherein the orally active insecticidal protein is at least part of a *Bacillus thuringiensis* δ endotoxin.

4. A plant gene expression cassette according to any one of claims 1 to 3, wherein the inducible promoter is derived from the *alc*A gene promoter.

5. A plant gene expression cassette according to any one of claims 1 to 4, wherein the inducible promoter is a chimeric promoter.

6. A plant cell containing a plant gene expression cassette according to any preceding claim.

7. A plant cell according to claim 6, wherein the plant gene expression cassette is stably incorporated in the plant's genome.

8. A plant tissue comprising a plant cell according to either of claims 6 and 7.

9. A plant comprising a plant cell according to either of claims 6 and 7.

10. A plant derived from a plant according to claim 9.

11. A seed derived from a plant according to either of claims 9 and 10.

12. A method of controlling insects comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 5.

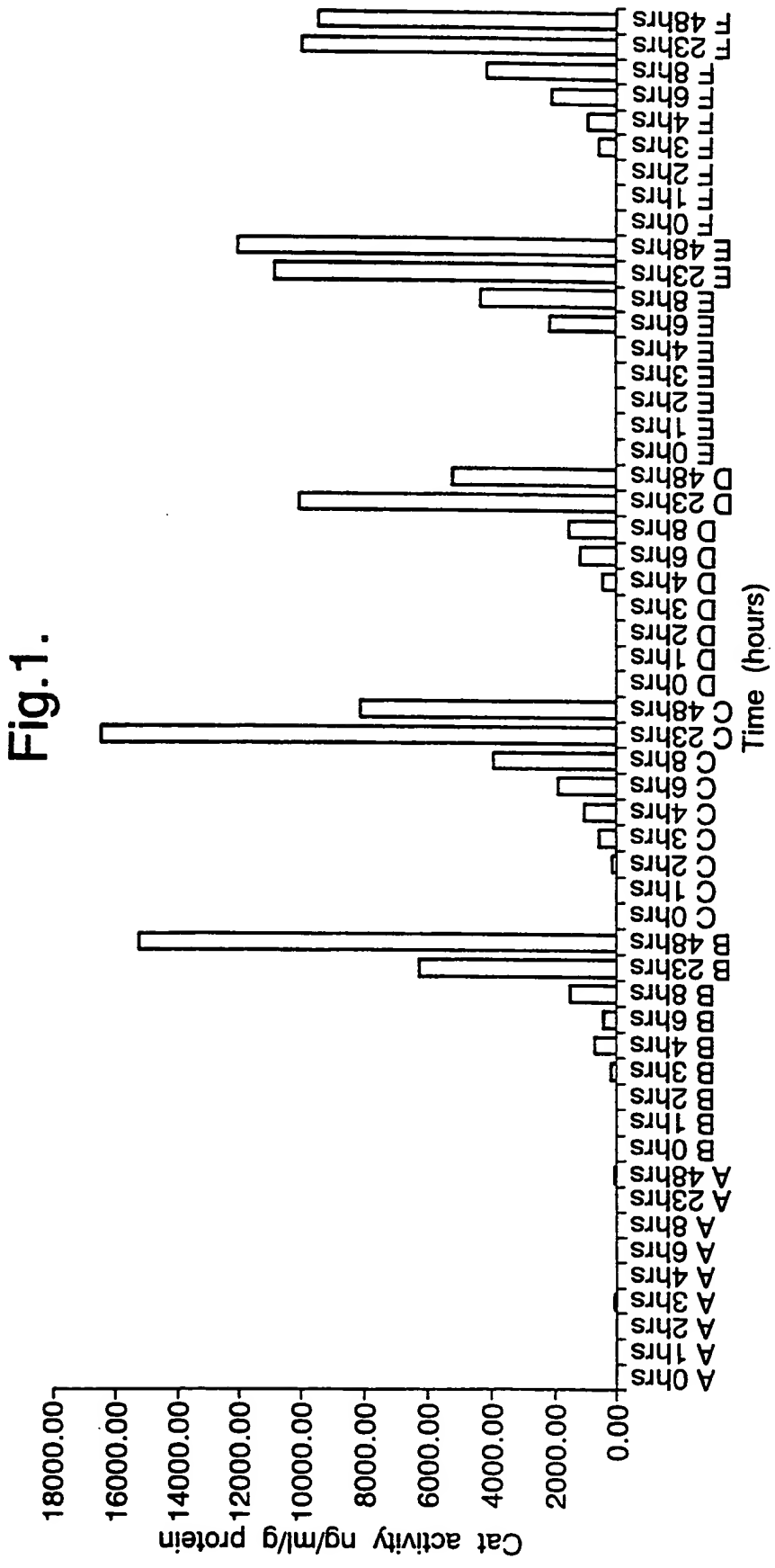

Cat activity ng/ml/g protein
18000.00
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
Time (hours)
A 0hrs
A 1hrs
A 2hrs
A 3hrs
A 4hrs
A 6hrs
A 8hrs
A 23hrs
A 48hrs
B 0hrs
B 1hrs
B 2hrs
B 3hrs
B 4hrs
B 6hrs
B 8hrs
B 23hrs
B 48hrs
C 0hrs
C 1hrs
C 2hrs
C 3hrs
C 4hrs
C 6hrs
C 8hrs
C 23hrs
C 48hrs
D 0hrs
D 1hrs
D 2hrs
D 3hrs
D 4hrs
D 6hrs
D 8hrs
D 23hrs
D 48hrs
E 0hrs
E 1hrs
E 2hrs
E 3hrs
E 4hrs
E 6hrs
E 8hrs
E 23hrs
E 48hrs
F 0hrs
F 1hrs
F 2hrs
F 3hrs
F 4hrs
F 6hrs
F 8hrs
F 23hrs
F 48hrs

Fig.1.


CAT activity ng/ml/g protein
350000.00
300000.00
250000.00
200000.00
150000.00
100000.00
50000.00
0.00
Chemical
1A
1C
2B
3A
3C
4B
5A
5C
6B
7A
7C
8B
9A
9C
10B
11A
11C
12B
13A
13C
14B
15A
15C
16B
17A
17C
20B
21A
21C
22B
23A
23C
ETOH B
acetone a
acetone c
dH20 B

Fig.2.

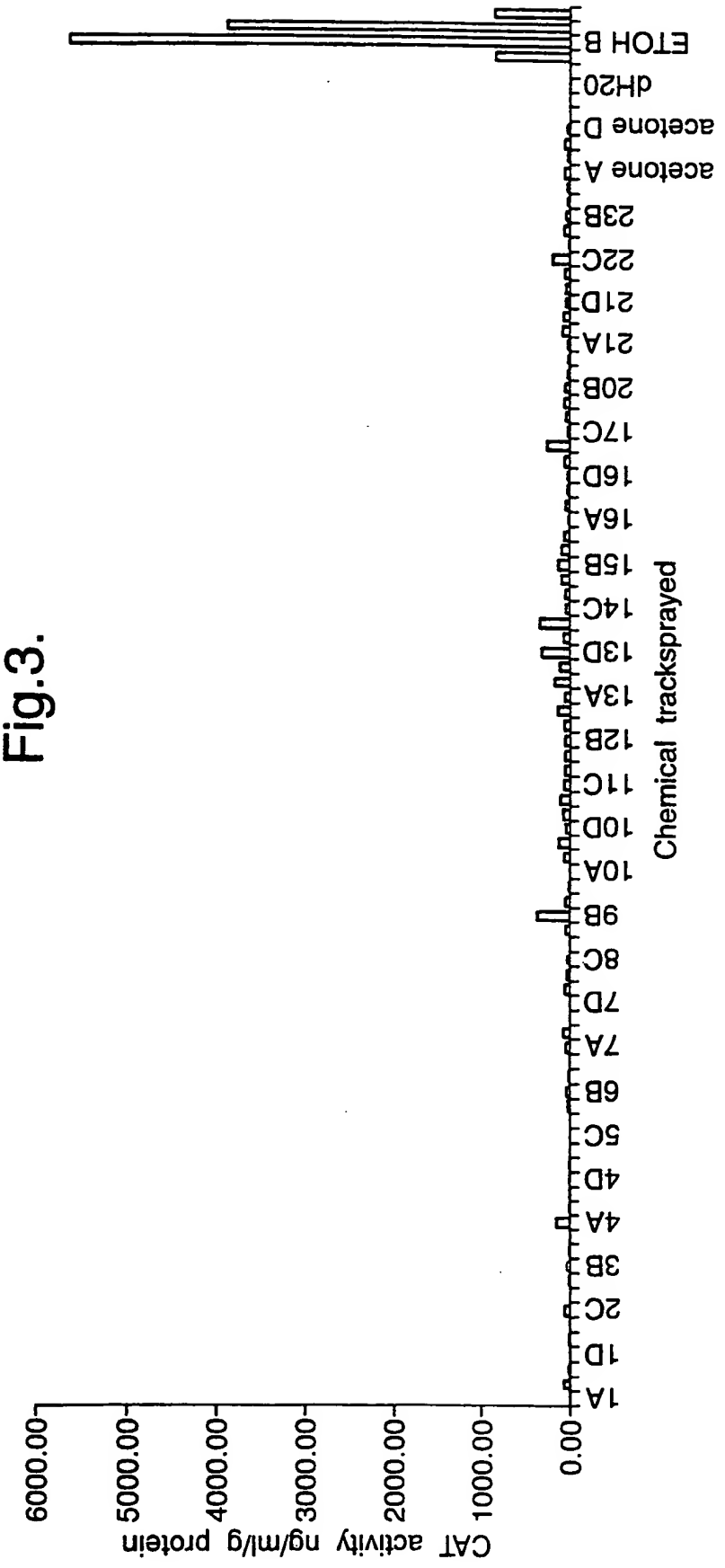

CAT activity ng/ml/g protein
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
Chemical tracksprayed
1A
1D
2C
3B
4A
4D
5C
6B
7A
7D
8C
9B
10A
10D
11C
12B
13A
13D
14C
15B
16A
16D
17C
20B
21A
21D
22C
23B
acetone A
acetone D
dH20
ETOH B

Fig.3.

Fig.4(1/2).

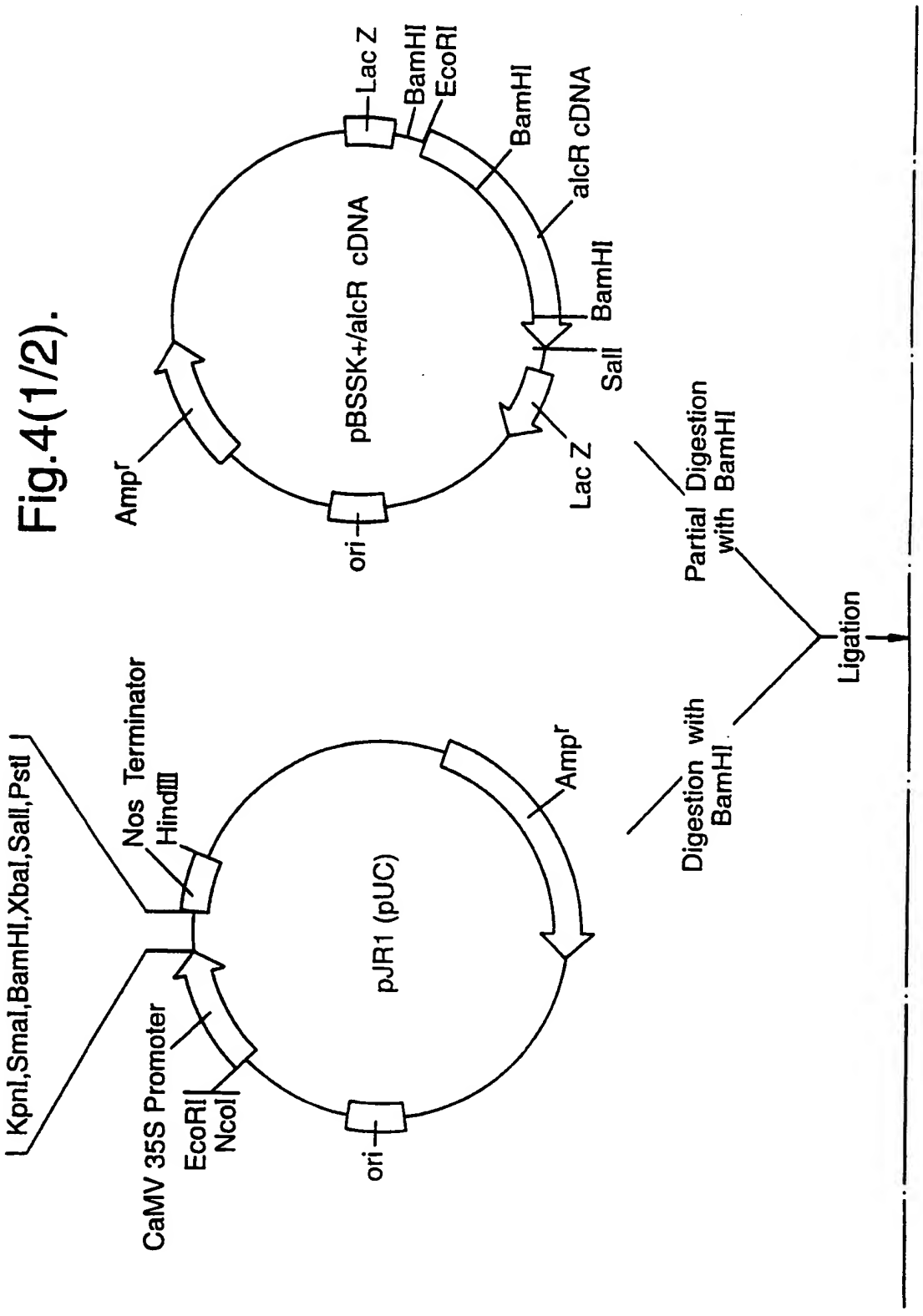

pBSSK+/alcR cDNA
Lac Z
BamHI
EcoRI
BamHI
alcR cDNA
BamHI
SalI
Lac Z
Amp^r
ori
Partial Digestion with BamHI
Digestion with BamHI
Ligation
pJR1 (pUC)
Amp^r
ori
Nos Terminator
HindIII
KpnI,SmaI,BamHI,XbaI,SalI,PstI
CaMV 35S Promoter
EcoRI
NcoI

Fig.4(2/2).

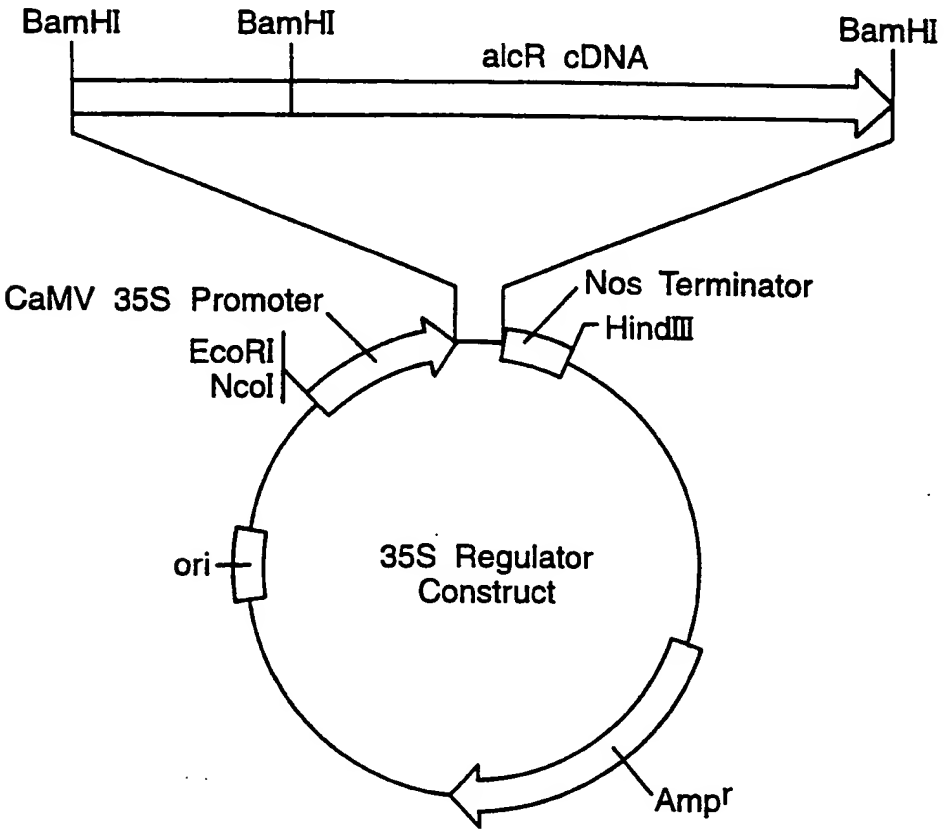
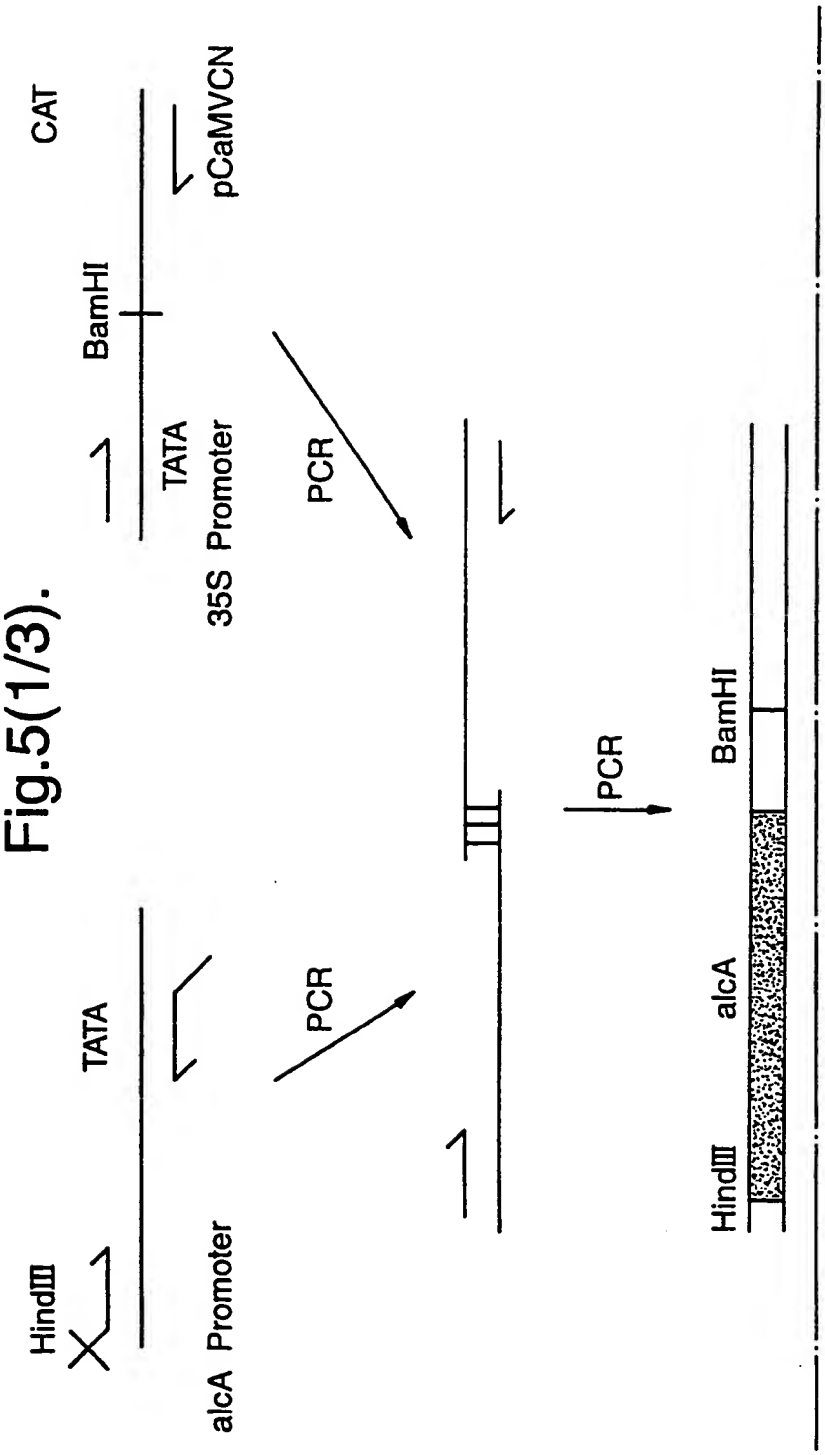

BamHI
BamHI
alcR cDNA
BamHI
CaMV 35S Promoter
EcoRI
NcoI
Nos Terminator
HindIII
ori
35S Regulator
Construct
Ampr

Fig.5(1/3).


HindIII
alcA Promoter
TATA
PCR
PCR
PCR
HindIII
alcA
BamHI
35S Promoter
TATA
BamHI
pCaMVCN
CAT

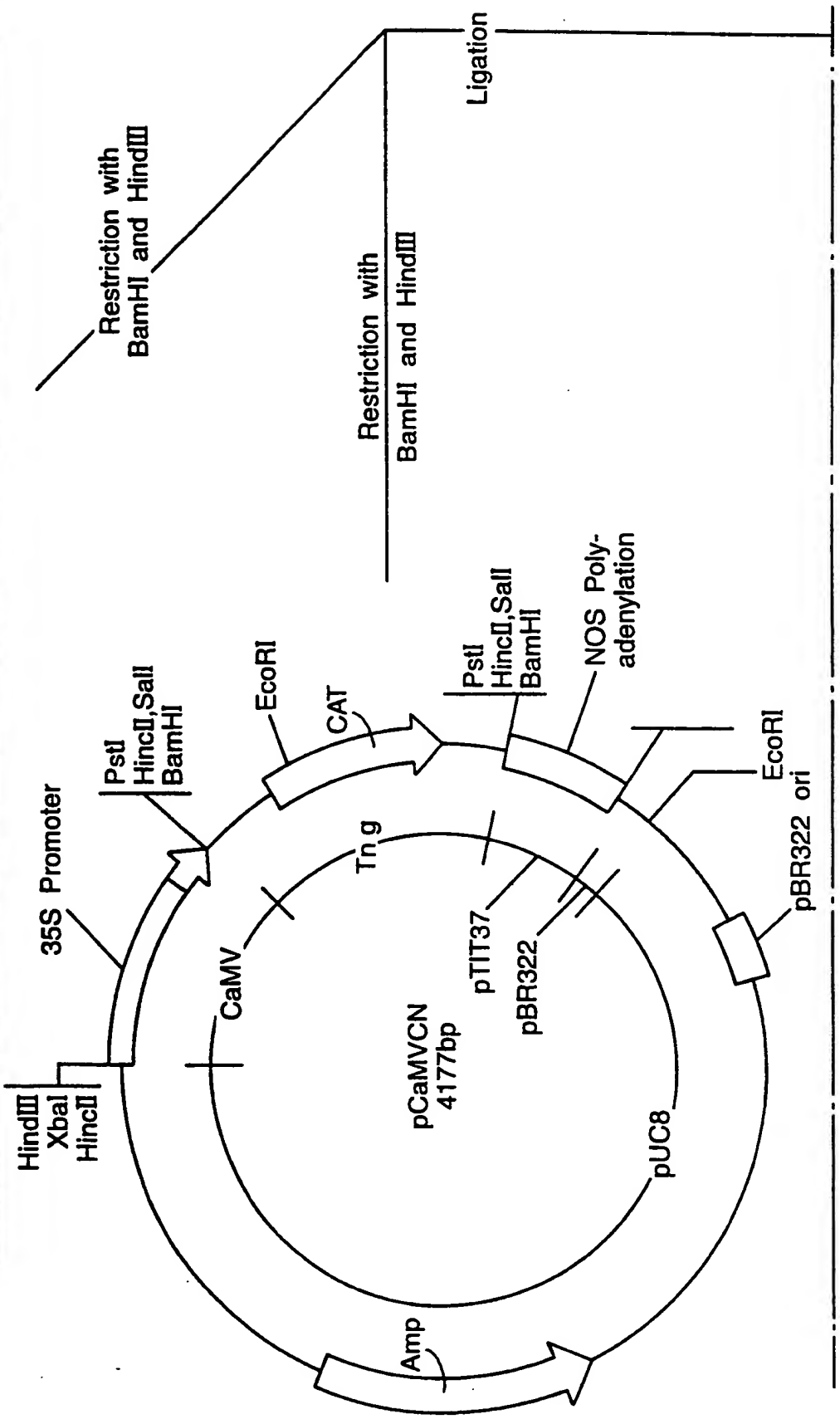

Ligation
Restriction with BamHI and HindIII
Restriction with BamHI and HindIII
PstI HincII,SalI BamHI
EcoRI
CAT
PstI HincII,SalI BamHI
NOS Poly-adenylation
EcoRI
pBR322 ori
35S Promoter
HindIII XbaI HincII
CaMV
Tn g
pTIT37
pBR322
pCaMVCN 4177bp
pUC8
Amp

Fig.5(2/3).

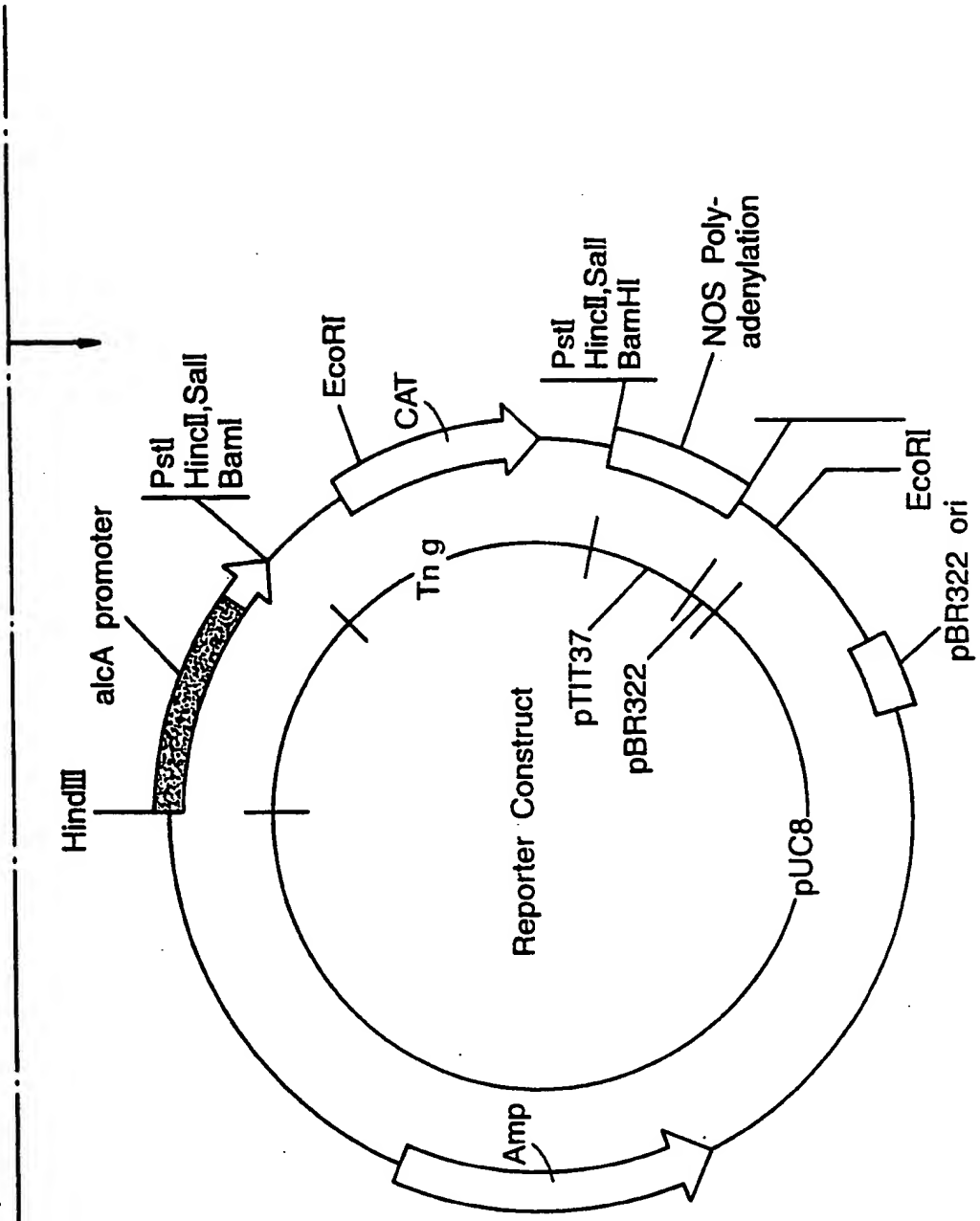
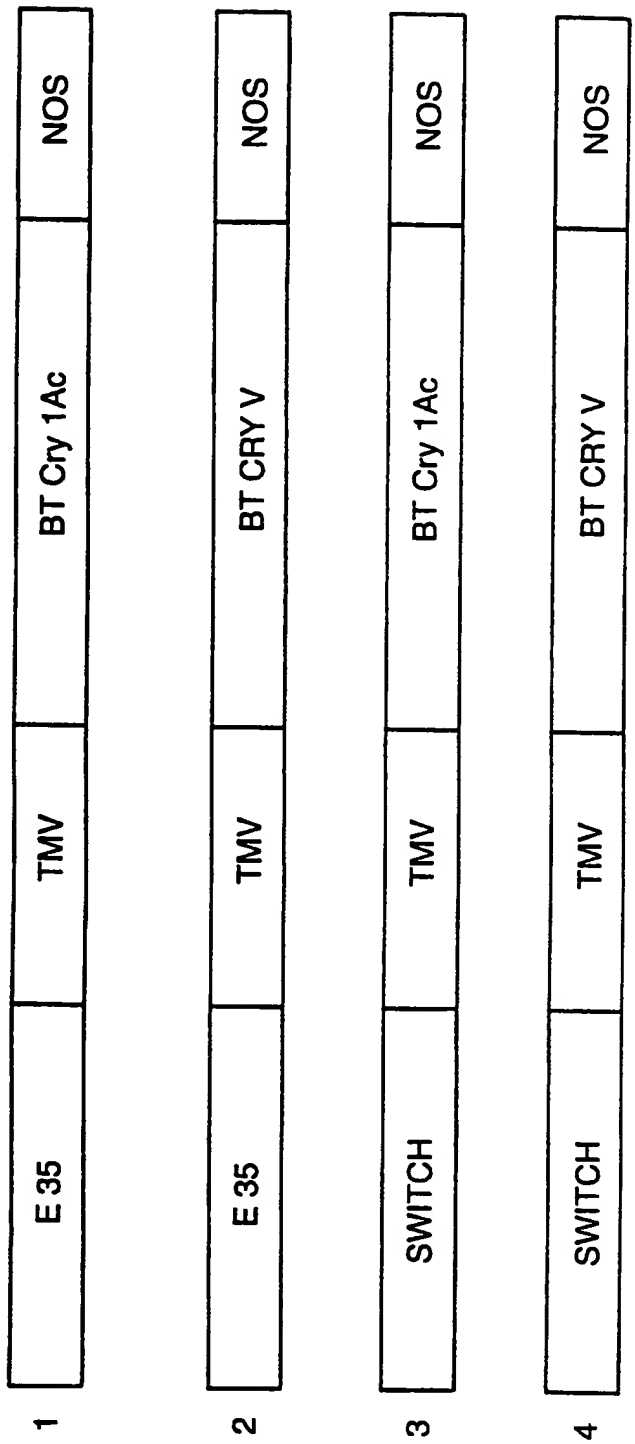

HindIII
alcA promoter
PstI
HincII,SalI
BamI
EcoRI
CAT
PstI
HincII,SalI
BamHI
NOS Poly-
adenylation
EcoRI
pBR322 ori
Tn g
pTIT37
pBR322
Reporter Construct
pUC8
Amp

Fig.5(3/3).

Fig.6.



E 35 = ENHANCED CaMV35

TMV = TMV ENHANCER

SWITCH - 35S alc R alc A promoter

Fig.7.

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
  1 AGATCTTAAA CAATGGCTAT GATTACGCCT AGCTTGCATG CCTGCATCCC GTACAACTGC
    TCTAGAATTT GTTACCGATA CTAATGCGGA TCGAACGTAC GGACGTAGGG CATGTTGACG

 61 CTGAGCAACC CGGAGGTGGA GGTGCTGGGC GGCGAGCGCA TCGAGACCGG CTACACGCCG
    GACTCGTTGG GCCTCCACCT CCACGACCCG CCGCTCGCGT AGCTCTGGCC GATGTGCGGC

121 ATCGACATCT CCCTGTCCCT CACCCAGTTC CTGCTGAGCG AGTTCGTCCC GGGCGCCGGC
    TAGCTGTAGA GGGACAGGGA GTGGGTCAAG GACGACTCGC TCAAGCAGGG CCCGCGGCCG

181 TTCGTGCTGG GCCTGGTGGA CATCATCTGG GGCATCTTCG GCCCGTCCCA GTGGGACGCC
    AAGCACGACC CGGACCACCT GTAGTAGACC CCGTAGAAGC CGGGCAGGGT CACCCTGCGG

241 TTCCTGGTGC AGATCGAGCA GCTGATCAAC CAGCGCATCG AGGAGTTCGC CCGCAACCAG
    AAGGACCACG TCTAGCTCGT CGACTAGTTG GTCGCGTAGC TCCTCAAGCG GGCGTTGGTC

301 GCCATCTCTA GACTGGAGGG CCTGAGCAAC CTGTACCAAA TCTACGCCGA GTCCTTCCGC
    CGGTAGAGAT CTGACCTCCC GGACTCGTTG GACATGGTTT AGATGCGGCT CAGGAAGGCG

361 GAGTGGGAGG CTGACCCGAC CAACCCGGCC CTGAGGGAGG AGATGCGCAT CCAGTTCAAC
    CTCACCCTCC GACTGGGCTG GTTGGGCCGG GACTCCCTCC TCTACGCGTA GGTCAAGTTG

421 GACATGAACT CCGCCCTGAC CACCGCCATC CCGCTGTTCG CCGTGCAGAA CTACCAGGTG
    CTGTACTTGA GGCGGGACTG GTGGCGGTAG GGCGACAAGC GGCACGTCTT GATGGTCCAC

481 CCGCTCCTGT CCGTGTACGT GCAGGCCGCC AACCTCCACC TGTCGGTCCT GAGGGACGTG
    GGCGAGGACA GGCACATGCA CGTCCGGCGG TTGGAGGTGG ACAGCCAGGA CTCCCTGCAC
```

Fig.7 (Cont).

```
  81 CAGGGCGTGT ACAGGACCCT CTCCTCCACC CTGTACCGCA GGCCCTTCAA CATCGGCATC
     GTCCCGCACA TGTCCTGGGA GAGGAGGTGG GACATGGCGT CCGGGAAGTT GTAGCCGTAG

 141 AACAACCAGC AGCTGTCCGT CCTGGACGGC ACCGAGTTCG CCTACGGCAC CTCCTCCAAC
     TTGTTGGTCG TCGACAGGCA GGACCTGCCG TGGCTCAAGC GGATGCCGTG GAGGAGGTTG

1201 CTGCCCTCCG CCGTATACAG GAAGAGCGGC ACCGTGGACT CCCTGGACGA GATCCCGCCG
     GACGGGAGGC GGCATATGTC CTTCTCGCCG TGGCACCTGA GGGACCTGCT CTAGGGCGGC

1261 CAGAACAACA ACGTCCCGCC GAGGCAGGGC TTCAGCCACC GCCTGAGCCA CGTGTCCATG
     GTCTTGTTGT TGCAGGGCGG CTCCGTCCCG AAGTCGGTGG CGGACTCGGT GCACAGGTAC

1321 TTCCGCTCCG GCTTCAGCAA CAGCAGCGTG AGCATCATCA GGGCCCCGAT GTTCTCCTGG
     AAGGCGAGGC CGAAGTCGTT GTCGTCGCAC TCGTAGTAGT CCCGGGGCTA CAAGAGGACC

1381 ATTCACCGCA GCGCCGAGTT CAACAACATC ATCGCCTCCG ACAGCATCAC CCAGATCCCG
     TAAGTGGCGT CGCGGCTCAA GTTGTTGTAG TAGCGGAGGC TGTCGTAGTG GGTCTAGGGC

1441 GCTGTGAAGG GCAACTTCCT GTTCAACGGC TCCGTGATCT CCGGCCCGGG CTTCACCGGC
     CGACACTTCC CGTTGAAGGA CAAGTTGCCG AGGCACTAGA GGCCGGGCCC GAAGTGGCCG

1501 GGCGACCTCG TCAGGCTGAA CAGCTCCGGC AACAACATCC AGAACCGCGG CTACATCGAG
     CCGCTGGAGC AGTCCGACTT GTCGAGGCCG TTGTTGTAGG TCTTGGCGCC GATGTAGCTC
```

Fig.7 i.

```
541  TCCGTGTTCG GCCAGCGCTG GGGCTTCGAC GCCGCCACCA TCAACAGCCG CTACAACGAC
     AGGCACAAGC CGGTCGCGAC CCCGAAGCTG CGGCGGTGGT AGTTGTCGGC GATGTTGCTG

601  CTGACCAGGC TGATCGGCAA CTACACCGAC TACGCCGTCC GCTGGTACAA CACCGGCCTG
     GACTGGTCCG ACTAGCCGTT GATGTGGCTG ATGCCGGCAGG CGACCATGTT GTGGCCGGAC

661  GAGCGCGTGT GGGGCCCGGA CTCTAGAGAC TGGGTCAGGT ACAACCAGTT CAGGCGCGAG
     CTCGCGCACA CCCCGGGCCT GAGATCTCTG ACCCAGTCCA TGTTGGTCAA GTCCGCGCTC

721  CTCACCCTCA CCGTGCTGGA CATCGTGGCC CTGTTCCCCA ACTACGACTC CAGGAGGTAC
     GAGTGGGAGT GGCACGACCT GTAGCACCGG GACAAGGGGT TGATGCTGAG GTCCTCCATG

781  CCCATCAGGA CCGTGAGCCA GCTGACCAGG GAAATCTACA CCAACCCCGT GCTGGAGAAC
     GGGTAGTCCT GGCACTCGGT CGACTGGTCC CTTTAGATGT GGTTGGGGCA CGACCTCTTG

841  TTCGACGGCA GCTTCCGCGG CAGCGCCCAG GGCATCGAGA GGAGCATCAG GAGCCCGCAC
     AAGCTGCCGT CGAAGGCGCC GTCGCGGGTC CCGTAGCTCT CCTCGTAGTC CTCGGGCGTG

901  CTCATGGACA TCCTGAACAG CATCACCATC TACACCGACG CCCACCGCGG CTACTACTAC
     GAGTACCTGT AGGACTTGTC GTAGTGGTAG ATGTGGCTGC GGGTGGCGCC GATGATGATG

961  TGGTCCGGCC ACCAGATCAT GGCCTCCCCC GTGGGCTTCT CCGGCCCGGA GTTCACCTTC
     ACCAGGCCGG TGGTCTAGTA CCGGAGGGGG CACCCGAAGA GGCCGGGCCT CAAGTGGAAG

1021 CCGCTGTACG GCACCATGGG CAACGCCGCC CCGCAGCAGA GGATCGTCGC CCAGCTCGGC
     GGCGACATGC CGTGGTACCC GTTGCGGCGG GGCGTCGTCT CCTAGCAGCG GGTCGAGCCG
```

Fig.7 (Cont i).

```
   61 GTCCCGATCC ACTTCCCGTC CACCTCCACC AGGTACAGGG TGAGGGTCCG CTACGCCTCC
      CAGGGCTAGG TGAAGGGCAG GTGGAGGTGG TCCATGTCCC ACTCCCAGGC GATGCGGAGG

 1621 GTGACCCCGA TCCACCTCAA CGTGAACTGG GGCAACTCCT CCATCTTCTC CAACACCGTC
      CACTGGGGCT AGGTGGAGTT GCACTTGACC CCGTTGAGGA GGTAGAAGAG GTTGTGGCAG

 1681 CCGGCCACCG CCACCTCCCT CGACAACCTC CAGTCCAGCG ACTTCGGCTA CTTCGAGAGC
      GGCCGGTGGC GGTGGAGGGA GCTGTTGGAG GTCAGGTCGC TGAAGCCGAT GAAGCTCTCG

 1741 GCCAACGCCT TCACCTCCTC CCTCGGCAAC ATCGTCGGCG TCAGGAACTT CTCCGGCACC
      CGGTTGCGGA AGTGGAGGAG GGAGCCGTTG TAGCAGCCGC AGTCCTTGAA GAGGCCGTGG

 1801 GCTGGCGTGA TCATCGACAG GTTCGAGTTC ATCCCGGTCA CCGCCACCCT CGAGCCGCCG
      CGACCGCACT AGTAGCTGTC CAAGCTCAAG TAGGGCCAGT GGCGGTGGGA GCTCGGCGGC

 1861 TAGGATCC
      ATCCTAGG
```

Tot number of bases is: 1868.
DNA sequence composition: 361 A; 702 C; 491 G; 314 T;

Sequence name: NCRY1AC.

==========

Fig.8.

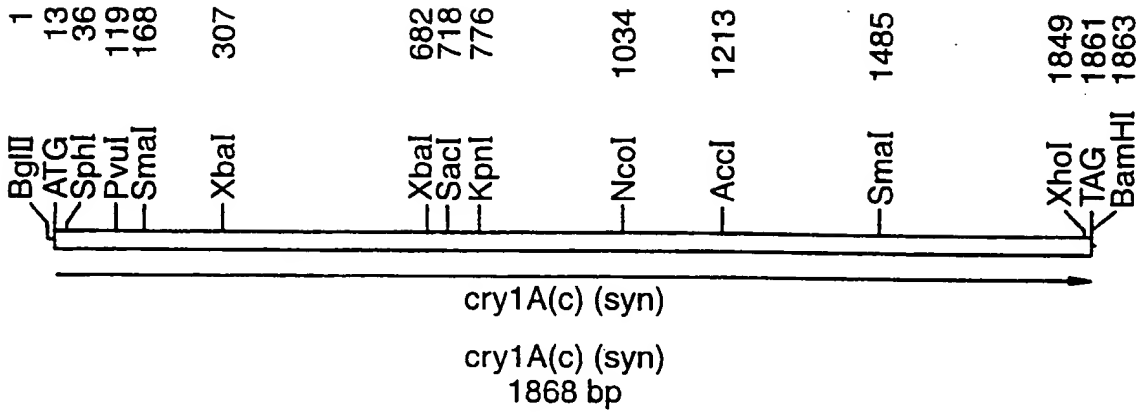
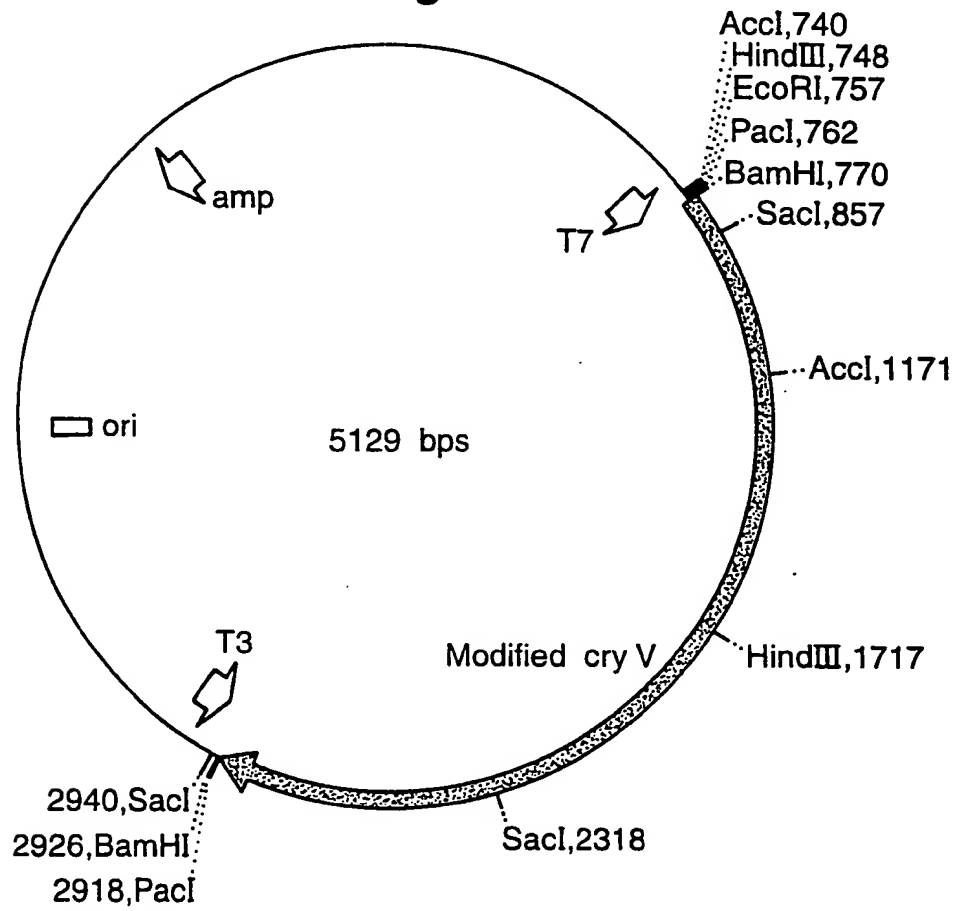


Fig.10.



The second version of the modified cry V gene in pBluescript (sk). NBI

Fig.9.

```
                10        20        30        40        50        60
                 |         |         |         |         |         |
  1 ATGAAGCTGA AGAACCAAGA CAAGCACCAA TCGTTCTCCA GCAACGCGAA AGTGGACAAG
    TACTTCGACT TCTTGGTTCT GTTCGTGGTT AGCAAGAGGT CGTTGCGCTT TCACCTGTTC

 61 ATCAGCACCG ACTCCCTGAA GAACGAGACC GACATCGAGC TCCAGAACAT CAACCACGAA
    TAGTCGTGGC TGAGGGACTT CTTGCTCTGG CTGTAGCTCG AGGTCTTGTA GTTGGTGCTT

121 GATTGCCTGA AGATGTCCGA GTACGAGAAC GTGGAGCCGT TCGTGAGCGC CTCCACCATC
    CTAACGGACT TCTACAGGCT CATGCTCTTG CACCTCGGCA AGCACTCGCG GAGGTGGTAG

181 CAGACCGGCA TCGGCATCGC GGGCAAGATC CTGGGTACCC TGGGCGTGCC GTTTGCCGGC
    GTCTGGCCGT AGCCGTAGCG CCCGTTCTAG GACCCATGGG ACCCGCACGG CAAACGGCCG

241 CAAGTGGCTA GCCTGTACAG CTTCATCCTC GGCGAGCTGT GGCCTAAGGG CAAGAACCAA
    GTTCACCGAT CGGACATGTC GAAGTAGGAG CCGCTCGACA CCGGATTCCC GTTCTTGGTT

301 TGGGAGATCT TCATGGAGCA CGTGGAGGAG ATCATCAACC AGAAGATTTC CACCTACGCC
    ACCCTCTAGA AGTACCTCGT GCACCTCCTC TAGTAGTTGG TCTTCTAAAG GTGGATGCGG

361 CGCAACAAGG CCCTTACCGA CCTGAAGGGC CTCGGCGACG CCCTGGCTGT CTACCACGAC
    GCGTTGTTCC GGGAATGGCT GGACTTCCCG GAGCCGCTGC GGGACCGACA GATGGTGCTG

421 TCCCTGGAGA GCTGGGTGGG CAACCGCAAC AACACGAGGG CCCGCAGCGT GGTGAAGAGC
    AGGGACCTCT CGACCCACCC GTTGGCGTTG TTGTGCTCCC GGGCGTCGCA CCACTTCTCG

481 CAGTACATCG CCCTGGAGCT GATGTTCGTG CAGAAGCTGC CGTCCTTCGC CGTGTCTGGT
    GTCATGTAGC GGGACCTCGA CTACAAGCAC GTCTTCGACG GCAGGAAGCG GCACAGACCA
```

Fig.9 (Cont).

```
     GGCGGCCATA AGCTGGAGTT CAGGACCATC GGCGGCACCC TCAACATCAG CACCCAAGGC
     CCGCCGGTAT TCGACCTCAA GTCCTGGTAG CCGCCGTGGG AGTTGTAGTC GTGGGTTCCG

1141 AGCACCAACA CCAGCATCAA CCCGGTCACC CTGCCCTTCA CCAGCCGCGA CGTGTACCGC
     TCGTGGTTGT GGTCGTAGTT GGGCCAGTGG GACGGGAAGT GGTCGGCGCT GCACATGGCG

1201 ACCGAGAGCC TGGCCGGCCT GAACCTGTTC CTGACCCAGC CCGTGAACGG CGTGCCCCGC
     TGGCTCTCGG ACCGGCCGGA CTTGGACAAG GACTGGGTCG GGCACTTGCC GCACGGGGCG

1261 GTGGACTTTC ACTGGAAGTT CGTGACCCAC CCGATCGCCA GCGACAACTT CTACTACCCC
     CACCTGAAAG TGACCTTCAA GCACTGGGTG GGCTAGCGGT CGCTGTTGAA GATGATGGGG

1321 GGCTACGCTG GCATCGGCAC CCAACTCCAG GACAGCGAGA ACGAGCTGCC GCCCGAGGCC
     CCGATGCGAC CGTAGCCGTG GGTTGAGGTC CTGTCGCTCT TGCTCGACGG CGGGCTCCGG

1381 ACCGGTCAGC CGAACTACGA GAGCTACAGC CACCGCCTGA GCCACATCGG CCTGATCTCC
     TGGCCAGTCG GCTTGATGCT CTCGATGTCG GTGGCGGACT CGGTGTAGCC GGACTAGAGG

1441 GCCTCCCACG TGAAGGCCCT GGTGTACTCC TGGACCCACC GCAGCGCCGA CCGCACCAAC
     CGGAGGGTGC ACTTCCGGGA CCACATGAGG ACCTGGGTGG CGTCGCGGCT GGCGTGGTTG

1501 ACCATCGAGC CGAACAGCAT CACGCAGATC CCGCTGGTGA AGGCCTTCAA CCTGAGCTCC
     TGGTAGCTCG GCTTGTCGTA GTGCGTCTAG GGCGACCACT TCCGGAAGTT GGACTCGAGG

1561 GGTGCTGCAG TGGTGCGCGG TCCAGGCTTC ACAGGCGGCG ACATCCTGCG CAGGACCAAC
     CCACGACGTC ACCACGCGCC AGGTCCGAAG TGTCCGCCGC TGTAGGACGC GTCCTGGTTG

1621 ACCGGCACCT TCGGCGACAT CCGCGTGAAC ATCAACCCCC CGTTCGCCCA GCGCTACAGG
     TGGCCGTGGA AGCCGCTGTA GGCGCACTTG TAGTTGGGGG GCAAGCGGGT CGCGATGTCC

1681 GTGAGGATCA GGTACGCCAG CACCACCGAC CTCCAGTTCC ACACCAGCAT CAACGGCAAG
     CACTCCTAGT CCATGCGGTC GTGGTGGCTG GAGGTCAAGG TGTGGTCGTA GTTGCCGTTC
```

Fig.9 i.

```
541  GAGGAGGTGC CCCTGCTGCC GATCTACGCC CAGGCCGCCA ACCTCCACCT CCTGCTCCTG
     CTCCTCCACG GGGACGACGG CTAGATGCGG GTCCGGCGGT TGGAGGTGGA GGACGAGGAC

601  CGCGACGCCA GCATCTTCGG CAAGGAGTGG GGCCTGTCCT CCAGCGAGAT CAGCACGTTC
     GCGCTGCGGT CGTAGAAGCC GTTCCTCACC CCGGACAGGA GGTCGCTCTA GTCGTGCAAG

661  TACAACAGGC AGGTGGAGCG CGCCGGCGAC TACAGCGACC ATTGCGTGAA GTGGTACAGC
     ATGTTGTCCG TCCACCTCGC GCGGCCGCTG ATGTCGCTGG TAACGCACTT CACCATGTCG

721  ACCGGCCTGA ACAACCTGAG GGGCACCAAC GCCGAGAGCT GGGTCCGCTA CAATCAGTTC
     TGGCCGGACT TGTTGGACTC CCCGTGGTTG CGGCTCTCGA CCCAGGCGAT GTTAGTCAAG

781  CGCCGCGACA TGACCCTGAT GGTGCTGGAC CTGGTGGCCC TGTTCCCGAG CTACGACACC
     GCGGCGCTGT ACTGGGACTA CCACGACCTG GACCACCGGG ACAAGGGCTC GATGCTGTGG

841  CAGATGTACC CGATCAAGAC CACCGCCCAG CTGACCCGCG AGGTGTACAC CGACGCCATT
     GTCTACATGG GCTAGTTCTG GTGGCGGGTC GACTGGGCGC TCCACATGTG GCTGCGGTAA

901  GGCACCGTGC ACCCGCACCC GAGCTTCACG AGCACCACCT GGTACAACAA CAACGCCCA
     CCGTGGCACG TGGGCGTGGG CTCGAAGTGC TCGTGGTGGA CCATGTTGTT GTTGCGGGGT

961  AGCTTCAGCG CCATCGAGGC CGCCGTGGTG CGCAACCCCC ACCTCCTGGA CTTCCTGGAG
     TCGAAGTCGC GGTAGCTCCG GCGGCACCAC GCGTTGGGGG TGGAGGACCT GAAGGACCTC

1021 CAGGTGACCA TCTACAGCCT GCTGAGCCGG TGGAGCAACA CGCAGTACAT GAACATGTGG
     GTCCACTGGT AGATGTCGGA CGACTCGGCC ACCTCGTTGT GCGTCATGTA CTTGTACACC
```

Fig.9 (Cont i).

```
1741 GCCATCAACC AGGGCAACTT CAGCGCCACC ATGAACCGCG GTGAGGACCT GGACTACAAG
     CGGTAGTTGG TCCCGTTGAA GTCGCGGTGG TACTTGGCGC CACTCCTGGA CCTGATGTTC

1801 ACCTTCCGCA CCGTGGGCTT CACCACCCCG TTCAGCTTCC TGGACGTGCA GAGCACCTTC
     TGGAAGGCGT GGCACCCGAA GTGGTGGGGC AAGTCGAAGG ACCTGCACGT CTCGTGGAAG

1861 ACCATCGGCG CCTGGAACTT CAGCAGCGGC AACGAGGTGT ACATCGACCG CATCGAGTTC
     TGGTAGCCGC GGACCTTGAA GTCGTCGCCG TTGCTCCACA TGTAGCTGGC GTAGCTCAAG

1921 GTGCCCGTGG AGGTGACCTA CGAGGCCGAG TACGACTTCG AGAAGGCCCA GGAGAAGGTC
     CACGGGCACC TCCACTGGAT GCTCCGGCTC ATGCTGAAGC TCTTCCGGGT CCTCTTCCAG

1981 ACCGCCCTGT TCACCAGCAC CAACCCGCGC GGCCTGAAGA CCGACGTGAA GGACTACCAC
     TGGCGGGACA AGTGGTCGTG GTTGGGCGCG CCGGACTTCT GGCTGCACTT CCTGATGGTG

2041 ATCGACCAGG TGAGCAACTT GGTGGAGTCC CTGAGCGACG AGTTCTACCT GGACGAGAAG
     TAGCTGGTCC ACTCGTTGAA CCACCTCAGG GACTCGCTGC TCAAGATGGA CCTGCTCTTC

2101 CGCGAGCTGT TCGAGATCGT GAAGTACGCC AAGCAGCTGC ACATCGAGCG CAACATGTAG
     GCGCTCGACA AGCTCTAGCA CTTCATGCGG TTCGTCGACG TGTAGCTCGC GTTGTACATC

2161 GATCC
     CTAGG
```

Total number of bases is: 2165.

Fig.11.

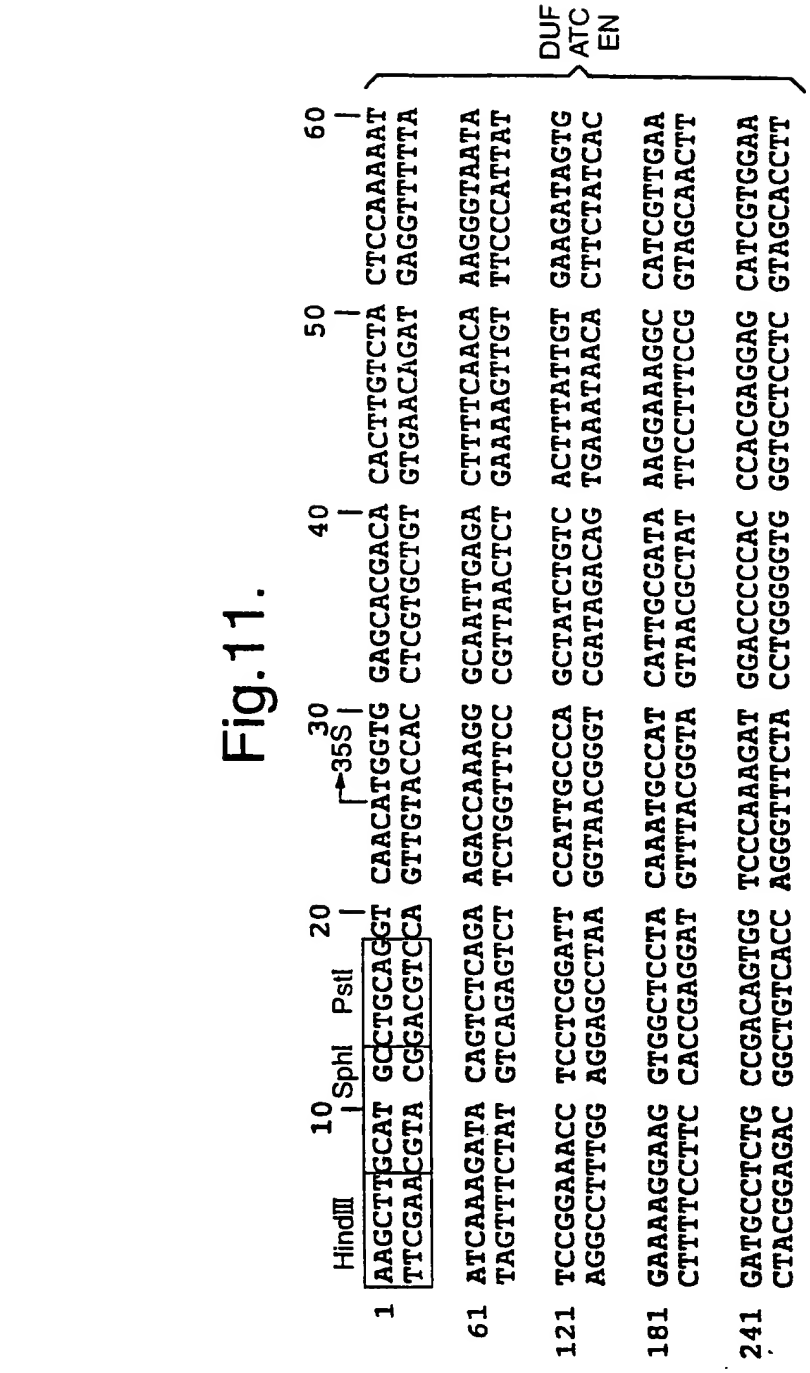
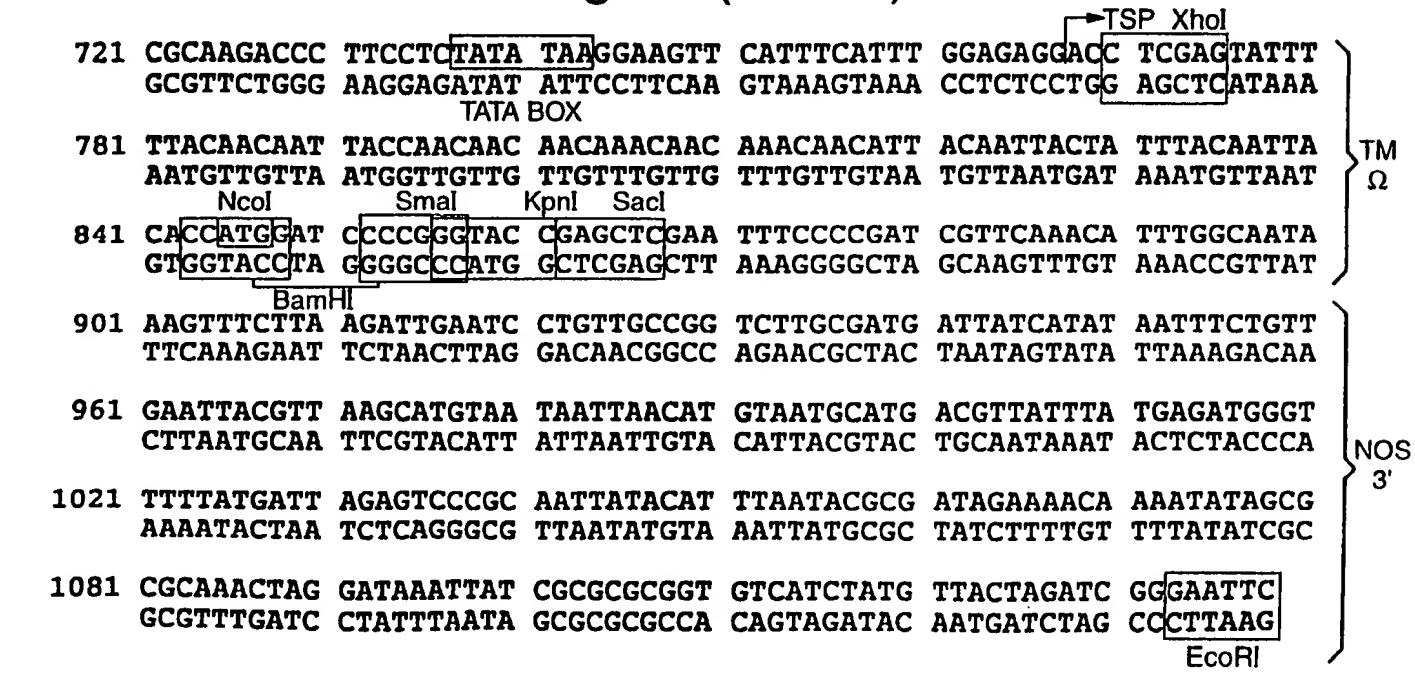

HindIII
SphI
PstI
35S
DUF ATC EN
1 AAGCTTGCATGCCTGCAGGT CAACATGGTG GAGCACGACA CACTTGTCTA CTCCAAAAAT
TTCGAACGTACGGACGTCCA GTTGTACCAC CTCGTGCTGT GTGAACAGAT GAGGTTTTTA
61 ATCAAGATA CAGTCTCAGA AGACCAAAGG GCAATTGAGA CTTTTCAACA AAGGGTAATA
TAGTTTCTAT GTCAGAGTCT TCTGGTTTCC CGTTAACTCT GAAAAGTTGT TTCCCATTAT
121 TCCGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC ACTTTATTGT GAAGATAGTG
AGGCCTTTGG AGGAGCCTAA GGTAACGGGT CGATAGACAG TGAAATAACA CTTCTATCAC
181 GAAAAGGAAG GTGGCTCCTA CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA
CTTTTCCTTC CACCGAGGAT GTTTACGGTA GTAACGCTAT TTCCTTTCCG GTAGCAACTT
241 GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA
CTACGGAGAC GGCTGTCACC AGGGTTTCTA CCTGGGGGTG GGTGCTCCTC GTAGCACCTT

Fig.11 (Cont).


35S PR
301 AAAAGAAGAC GTTCCAACCA CGTCTTCAAA GCAAGTGGAT TGATGTGATA ACATGGTGGA
TTTTCTTCTG CAAGGTTGGT GCAGAAGTTT CGTTCACCTA ACTACACTAT TGTACCACCT
361 GCACGACACA CTTGTCTACT CCAAAAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC
CGTGCTGTGT GAACAGATGA GGTTTTTATA GTTTCTATGT CAGAGTCTTC TGGTTTCCCG
421 AATTGAGACT TTTCAACAAA GGGTAATATC CGGAAACCTC CTCGGATTCC ATTGCCCAGC
TTAACTCTGA AAAGTTGTTT CCCATTATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTCG
481 TATCTGTCAC TTTATTGTGA AGATAGTGGA AAAGGAAGGT GGCTCCTACA AATGCCATCA
ATAGACAGTG AAATAACACT TCTATCACCT TTTCCTTCCA CCGAGGATGT TTACGGTAGT
541 TTGCGATAAA GGAAAGGCCA TCGTTGAAGA TGCCTCTGCC GACAGTGGTC CCAAAGATGG
AACGCTATTT CCTTTCCGGT AGCAACTTCT ACGGAGACGG CTGTCACCAG GGTTTCTACC
601 ACCCCCACCC ACGAGGAGCA TCGTGGAAAA AAGAAGACGT TCCAACCACG TCTTCAAAGC
TGGGGGTGGG TGCTCCTCGT AGCACCTTTT TTCTTCTGCA AGGTTGGTGC AGAAGTTTCG
EcoRV
661 AAGTGGATTG ATGTGATATC TCCACTGACG TAAGGGATGA CGCACAATCC CACTATCCTT
TTCACCTAAC TACACTATAG AGGTGACTGC ATTCCCTACT GCGTGTTAGG GTGATAGGAA
Promoter

Fig.11 (Cont i).

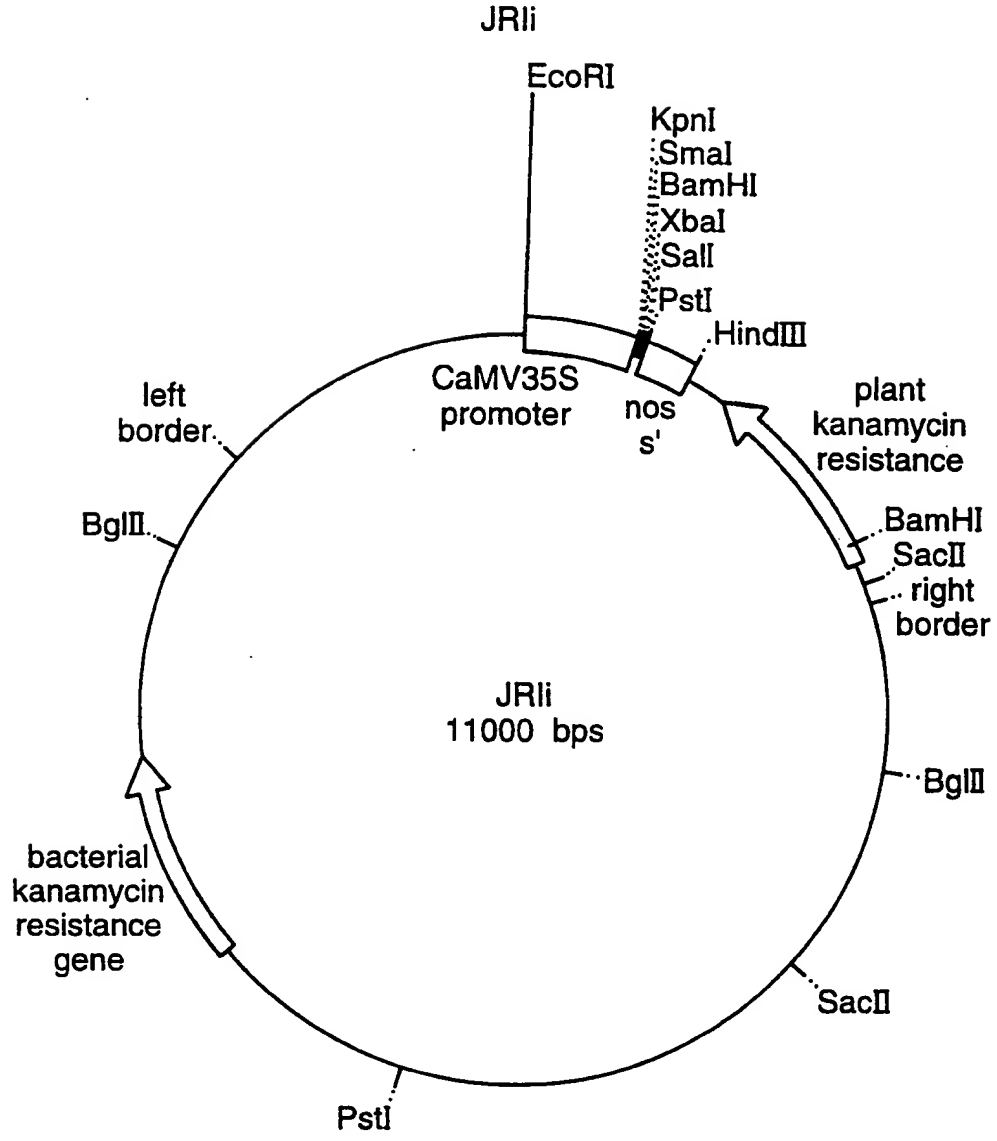



```
721 CGCAAGACCC TTCCTCTATA TAAGGAAGTT CATTTCATTT GGAGAGGACC TCGAGTATTT
    GCGTTCTGGG AAGGAGATAT ATTCCTTCAA GTAAAGTAAA CCTCTCCTGG AGCTCATAAA

781 TTACAACAAT TACCAACAAC AACAAACAAC AAACAACATT ACAATTACTA TTTACAATTA
    AATGTTGTTA ATGGTTGTTG TTGTTTGTTG TTTGTTGTAA TGTTAATGAT AAATGTTAAT

841 CACCATGGAT CCCCGGGTAC CGAGCTCGAA TTTCCCCGAT CGTTCAAACA TTTGGCAATA
    GTGGTACCTA GGGGCCCATG GCTCGAGCTT AAAGGGGCTA GCAAGTTTGT AAACCGTTAT

901 AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCGATG ATTATCATAT AATTTCTGTT
    TTCAAAGAAT TCTAACTTAG GACAACGGCC AGAACGCTAC TAATAGTATA TTAAAGACAA

961 GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCATG ACGTTATTTA TGAGATGGGT
    CTTAATGCAA TTCGTACATT ATTAATTGTA CATTACGTAC TGCAATAAAT ACTCTACCCA

1021 TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGCG ATAGAAAACA AAATATAGCG
     AAAATACTAA TCTCAGGGCG TTAATATGTA AATTATGCGC TATCTTTTGT TTTATATCGC

1081 CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTACTAGATC GGGAATTC
     GCGTTTGATC CTATTTAATA GCGCGCGCCA CAGTAGATAC AATGATCTAG CCCTTAAG
```

Total number of bases is: 1138.
DNA sequence composition: 370 A; 253 C; 234 G; 281 T; 0 OTHER;

Sequence name:PMJB1

Fig.12.


JRIi
EcoRI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
HindIII
CaMV35S promoter
nos s'
plant kanamycin resistance
BamHI
SacII
right border
BglII
SacII
PstI
bacterial kanamycin resistance gene
BglII
left border
JRIi
11000 bps